PLANT SANCTIONS AND POLLINATOR BEHAVIOUR IN THE FIG TREE – FIG WASP MUTUALISM

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PLANT SANCTIONS AND POLLINATOR BEHAVIOUR IN THE FIG TREE – FIG WASP MUTUALISM

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Theory predicts that mutualisms should be vulnerable to invasion by cheaters, yet mutualistic interactions are both ancient and diverse. When cooperation is costly, what prevents one partner from reaping the benefits of the interaction without paying the costs?

In Chapter One, we examined factors affecting mutualism stability in six fig tree – fig wasp species pairs. We experimentally compared the fitness of wasps that did or did not perform their most basic mutualistic service, pollination. We found host sanctions that reduced the fitness of non-pollinating wasps in all derived, actively pollinated fig species (where wasps expend time and energy pollinating), but not in the basal, passively pollinated fig species (where wasps do not). We further screened natural populations of pollinators for wasp individuals that did not carry pollen. Pollen-free wasps occurred only in actively pollinating wasp species, and their prevalence was negatively correlated with the sanction strength of their host species. Our findings suggest that sanctions are critical for long term mutualism stability when providing benefits to a host is costly.

In Chapter Two, we examined the precision of host sanctions. If multiple

symbionts interact with each host or host module, sanctions may not be precise enough to effectively punish cheating symbionts individually. Such lack of precision could greatly reduce the effectiveness of sanctions. Combining field experiments and molecular methods, we show that offspring reducing sanctions in *Ficus nymphaefolia* act on the fig level, not on the level of individual flowers within a fig. Such imprecise modular sanctions allow pollen-free wasps to free-ride in figs with multiple foundresses.

In Chapter Three, I use mathematical models parameterized with empirical data to examine what mechanisms are most likely to maintain the behavioural polymorphism in fig wasps. Is being a pollen-free wasp adaptive, or is this morph maladaptive but repeatedly introduced through mutations? The large differences across fig species in the strength and likelihood of sanctions lead to different mechanisms being likely for maintaining the wasp polymorphisms in these closely related wasp species.

BIOGRAPHICAL SKETCH

Karin Charlotte Jandér was born and pursued her early education in Sweden. As an undergraduate at Uppsala University she studied medicine and biology in parallel, eventually choosing medicine and graduating with an M.D. from Uppsala University in 1999. She completed her residency at Mora Lasarett, Sweden, and became a fully licensed doctor in 2000. In 2001 she returned to biology, spent six months as a research assistant in a canopy ecology project in Monteverde, Costa Rica, then re-enrolled at Uppsala University and completed a Master's degree in Biology in 2003, with a particular focus on behaviour, ecology and evolution. Her studies included a year as an exchange student at James Cook University in Townsville & Cairns, Australia, 2002, where she enjoyed studying tropical ecology on site. Charlotte's Master's project took her to the Smithsonian Tropical Research Institute (STRI) in Panama, where Edward Allen Herre introduced her to the amazing world of fig trees and fig wasps. During her Master's project she particularly focused on fig wasp behaviour, but also discovered aspects of fig biology that formed the seeds of her Ph.D. project.

Charlotte enrolled at Cornell University in August 2004, advised jointly by Paul W. Sherman and H. Kern Reeve. She continued to enjoy extensive field seasons at STRI in Panama. Charlotte was a visiting scholar at University of California, Berkeley 2006-2010, with Ellen L. Simms, and there learned how to apply molecular methods to her fig wasps. In 2010 she returned to Uppsala University as a visiting scholar with Jon Ågren while finishing her thesis. Charlotte taught undergraduate and

graduate level courses focusing on ecology, evolution and behaviour at Cornell University, U.C. Berkeley and Uppsala University. Her research interests include the ecology and evolution of mutualisms, with a particular focus on mechanisms that maintain mutualism stability and prevent symbiont cheating. Charlotte and her research assistants study fig tree sanctions and pollinator behaviour using surveys of natural populations, manipulative experiments in the field, greenhouse and laboratory, behavioural observations in the field and laboratory, mathematical models and molecular methods.

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This thesis would not have been possible without the hard work and dedication

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CHAPTER 1

HOST SANCTIONS AND POLLINATOR CHEATING IN THE FIG TREE - FIG WASP MUTUALISM[1]

Abstract

Theory predicts that mutualisms should be vulnerable to invasion by cheaters, yet mutualistic interactions are both ancient and diverse. What prevents one partner from reaping the benefits of the interaction without paying the costs? Using field experiments and observations, we examined factors affecting mutualism stability in six fig tree – fig wasp species pairs. We experimentally compared the fitness of wasps that did or did not perform their most basic mutualistic service, pollination. We found host sanctions that reduced the fitness of non-pollinating wasps in all derived, actively pollinated fig species (where wasps expend time and energy pollinating), but not in the basal, passively pollinated fig species (where wasps do not). We further screened natural populations of pollinators for wasp individuals that did not carry pollen ("cheaters"). Pollen-free wasps occurred only in actively pollinating wasp species, and their prevalence was negatively correlated with the sanction strength of their host species. Combined with previous studies, our findings suggest that 1) mutualisms can show co-evolutionary dynamics analogous to those of "arms-races" in overtly antagonistic interactions, 2) sanctions are critical for long

[1] Reprinted from Proceedings of the Royal Society B, Vol. 277, Jandér, K.C. and Herre, E.A., Host sanctions and pollinator cheating in the fig tree – fig wasp mutualism, pp. 1481-1488, © 2010 The Royal Society. Reprinted with permission from the Royal Society.

term mutualism stability when providing benefits to a host is costly, and 3) there are general principles that help maintain cooperation both within and among species.

1. Introduction

From the mycorrhizal fungi that are essential for the growth of most forest trees to the intestinal bacteria that provide nutrients we otherwise could not access, mutualisms are of fundamental ecological and evolutionary importance (Herre *et al.* 1999). Despite this, the evolution and maintenance of mutualisms remains a largely unsolved puzzle (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1991; Sachs *et al.* 2004; West *et al.* 2007). In a mutualistic system where the partners trade costly services, individuals that reap the benefits of the interactions without paying the cost of providing service to the mutualist (i.e. "cheaters") are expected to realize higher fitness than non-cheaters, and increase in their relative frequency. Therefore, the long-term stability of the mutualism likely requires mechanisms that limit or prevent cheating. Some studies have documented the existence of host "sanctions" that lower the fitness of uncooperative symbionts (Nefdt 1989; Pellmyr & Huth 1994; Richter & Weis 1995; Wilson & Addicott 1998; Huth & Pellmyr 2000; Jousselin & Kjellberg 2001; Kiers *et al.* 2003; Edwards *et al.* 2006; Kiers *et al.* 2006; Simms *et al.* 2006; Tarachai *et al.* 2008; Bever *et al.* 2009; Heath & Tiffin 2009). For example, both cultivated and wild legumes can selectively suppress rhizobial strains that feed off of plants but are inefficient at fixing nitrogen (Kiers *et al.* 2003; Kiers *et al.* 2006; Simms *et al.* 2006), and yuccas differentially abort inflorescences that are relatively underpollinated or overexploited by their pollinating moth (Pellmyr & Huth 1994; Richter & Weis 1995; Wilson & Addicott 1998; Huth & Pellmyr 2000). However, no study has examined

variation in sanction strength across related host species. Further, although distinct cheating/parasitic species are known to exploit many mutualisms (Sachs & Simms 2006), examples of cheating individuals within a mutualistic species are rare (Bronstein 2001). Here we use the fig tree – fig wasp system to test whether cheating levels in symbionts are related to sanction strength.

The fig tree – fig wasp system is well suited for studying the evolution and stability of mutualisms. This mutualism is both ancient (80 MYA) and diverse (>700 fig species) (Machado *et al.* 2001; Rønsted *et al.* 2005), and each fig species is generally pollinated by one or a few highly host-specific wasp species (Molbo *et al.* 2003; Haine *et al.* 2006). Each partner is totally dependent on the other for reproductive success, and lifetime reproductive success of the wasp is easily measured (Herre 1989). Fig flowers are located on the inside of the distinctive, enclosed inflorescences that define the genus *Ficus* (formally syconia; hereafter figs). Pollination is completely dependent on having one or more pollen-bearing female fig wasp (foundress) enter each fig and pollinate the flowers. Fig wasps, in turn, can only oviposit and reproduce in fig flowers.

Wasps generally pollinate both the flowers in which they oviposit, and those that do not receive eggs (Jousselin & Kjellberg 2001; Jandér 2003), then die inside the fig. In monoecious fig species (the roughly 50% of fig species that produce seeds and wasps in the same fig, as opposed to on different trees (dioecious)), each flower produces either a seed or is transformed into a gall that hosts a single wasp larva, causing a direct trade-off between producing fig seeds or wasp offspring (Verkerke 1989; Herre & West 1997). When mature, the wasp offspring mate and

females gather pollen within their natal fig before they fly off in search for a new receptive fig tree. Thus, monoecious fig trees depend on the foundress generation to pollinate their own flowers and initiate seed production (thereby realizing "female function") and on the females of the offspring generation to use their pollen to initiate seed production in another tree (thereby realizing "male function").

There are two distinct pollination syndromes in figs that demand different levels of effort from the pollinating wasps: 1) passive pollination, the ancestral condition, and 2) active pollination, the more derived condition (Jousselin *et al.* 2003b). Passively pollinated fig species produce numerous, large male flowers which release abundant pollen onto the wasps as they leave the fig to disperse. Typically, male to female flower ratios range from 0.25 to 1, and pollen to ovule ratios can be as high as 44,000 to 1 (Cruden 1997; Kjellberg *et al.* 2001). Therefore, in these fig species, trees invest considerable resources in producing abundant pollen, and no aspect of pollen transfer relies on specialized wasp behaviour.

In contrast, actively pollinated fig species produce relatively few, small male flowers. Male to female flower ratios range from 0.01 to 0.15, and pollen to ovule ratios are generally 5 to 10 times lower than that in passively pollinated species (Cruden 1997; Kjellberg *et al.* 2001). In these fig species, pollen transfer is completely dependent on specialized wasp morphology and pollination behaviour. Female wasps search for the male flowers inside their natal fig**,** gather pollen using their front legs, and store it in specialized thoracic pollen pockets (Galil & Snitzer-Pasternak 1970; Frank 1984). When wasps reach a receptive fig, they oviposit, then use their front legs to transfer pollen grains to the stigmas (Galil & Eisikowitch 1969; Frank 1984).

Overall, 2-5% of the wasps' total time within a receptive fig is spent actively depositing pollen (Jandér 2003, KCJ unpublished). Active pollination thus appears to be beneficial for trees as less pollen production is required, but it requires time and energy from the short-lived wasps. Previous studies suggest that wasps ovipositing in pollinated figs produce more offspring than wasps ovipositing in un-pollinated figs (reviewed in Herre *et al.* 2008).

Here, we use field experiments and observations to examine factors that affect the host-pollinator relationships in six monoecious fig species – four actively pollinated (where wasps actively expend time and energy pollinating) and two passively pollinated (where pollination is a byproduct of the wasps' activities). First, we experimentally measured sanction strength in the respective fig species by relating total lifetime reproductive success for a single foundress wasp to whether or not the wasp was carrying pollen. Second, for the different wasp species we estimated the likelihood that a wasp would be a single foundress – the situation in which a cheating wasp would be most fully exposed to any host sanctions. Third, we screened natural populations of pollinator wasps for wasp individuals that did not carry pollen ("cheaters*").* We thus were able to examine 1) whether host sanctions were present in these fig species, and if that was related to the pollination syndrome (passive or active), and 2) whether pollinator cheating levels were related to the strength of sanctions or the likelihood of being a single foundress.

2. Materials and Methods

Study system

We studied natural populations of trees and wasps near the Panama Canal, Republic of Panama. The passively pollinated fig species represent the most basal of all fig lineages, subgenus *Pharmacosycea*, section *Pharmacosycea*: *Ficus maxima* and *F. insipida* (Herre *et al.* 1996; Machado *et al.* 2001; Jousselin *et al.* 2003b; Rønsted *et al.* 2005). The actively pollinated fig species all belong to the more derived subgenus *Urostigma*, section *Americana: F. citrifolia, F. nymphaefolia, F. obtusifolia* and *F. popenoei*. The respective pollinator wasp species and mean number of female flowers are specified in table S1 (appendix chapter 1). For simplicity, we will here use the fig species name as a proxy also for its associated wasp species.

Pollen exclusion experiment

For each fig tree-pollinator species-pair, we experimentally produced pollen-carrying (P+) and artificially pollen-free (AP-) wasps, and introduced one wasp into each fig to produce pollinated (P+) and unpollinated (P-) figs (Jousselin *et al.* 2003a). We quantified two components of fig sanctions that strongly influence wasp fitness: 1) the proportion of P- and P+ figs that the tree aborted prior to maturation (fig abortion leads to 100% mortality of the enclosed wasp larvae), and 2) the reproductive success of AP- and P+ wasps in the un-aborted figs.

We first surveyed several hundred fig trees to match pairs of nearly ripe trees (producing wasps) with nearly receptive conspecific experimental trees. We prevented uncontrolled pollination by enclosing multiple twigs on each pre-receptive tree in mesh bags. To obtain artificially pollen-free (AP-) wasps of the pollinator species, we gathered nearly ripe figs from different,

conspecific trees, and opened the figs when male wasps were mating with the females, but when females were still within their galls. We removed all male flowers to prevent female wasps from accessing pollen when they emerged. Control wasps with pollen loads (P+) emerged normally from ripe figs into mesh-covered vials (Jousselin *et al.* 2003a).

When figs on the experimental tree were receptive, a single AP- or P+ female wasp was introduced into each randomly assigned fig. To determine the effects of no foundress (F- ; i.e. no oviposition and no pollination), some figs were left without any wasp entering. All experimental figs on each tree were approximately the same size, and when possible paired figs were used for the P+ and P- treatments. We then re-bagged the twigs to prevent attacks by parasites. During the weeks following the experimental introductions we collected any aborted figs and checked them - figs in which a foundress had been introduced but had not successfully entered the internal cavity of the fig were excluded from the study. The majority of aborted but entered figs showed macroscopic signs of gall development, indicating wasp oviposition. At the end of the experiment, we collected the non-aborted figs just before wasps emerged, so that wasps could emerge in vials and be counted. In a few cases where wasps had already emerged, we counted empty wasp galls to quantify the number of offspring. Experimental figs on *F. maxima* tree # 2 were lost due to a neighbouring tree fall a few days before maturation (well after any abortions); hence abortions could be assessed but wasp offspring could not be counted. We counted seeds in each fig to confirm a successful treatment. In some cases there were a few seeds in the P- treatment (usually less than 1% of seeds in the P+ treatment). These figs were included in the P- treatment in the analyses; the results did not change if only figs with zero seeds were included.

To enable direct comparisons across species, we calculated the following values for each tree:

M_{P-} = The proportion of figs in the P- treatment that matured (did not abort).

M_{P+} = The proportion of figs in the P+ treatment that matured (did not abort).

O_{P-} = The mean number of wasp offspring in P- figs that matured.

O_{P+} = The mean number of wasp offspring in P+ figs that matured.

$M_R = M_{P-}/ M_{P+}$ = The relative proportion P- figs that matured.

$O_R = O_{P-}/ O_{P-}$ = The relative number of offspring in un-aborted P- figs.

$W_R = M_R * O_R$ = The relative fitness of a single foundress P- wasp. Assuming P- and P+ foundresses laid similar amounts of eggs (appendix chapter 1), this is equivalent to the relative survival of P- eggs to P+ eggs (Wenseleers & Ratnieks 2006).

Thus, each tree produced a single value of M_R, O_R, and W_R, and we compared these variables across species using ANOVAS. We square-root transformed W_R to meet the assumption of homogeneity of variances for the ANOVA, but we used untransformed data for graphs and magnitude comparisons. Performing the ANOVA on untransformed data did not change the results.

Proportion single foundress wasps in each species

To estimate the proportion of wasps associated with each species that are likely to be single foundresses, we collected figs within a week after pollinator arrival (with few exceptions >100 figs per crop; number of crops: *F. pop*. 12, *F. nym*. 5, *F. cit*. 6, *F. obt*. 8), and counted the number of dead foundress wasps in each fig. We then calculated the proportion of wasps that were single foundresses (e.g. if 50% of figs had one foundress and 50% had two, then 1/3 of

wasps were single foundresses), and tested whether this proportion differed across species using a generalized linear model with binomial errors, a logit link, and an overdispersion parameter, using single foundress wasps (out of total number of wasps) for each crop as the response variable, and species as the explanatory variable; contrasts were pairwise and sequential Bonferroni corrected. Results did not change if we instead used a Kruskal Wallis test to examine whether the proportion of single foundress wasps differed across species.

Prevalence of pollen-free wasps in natural populations

Unmanipulated, naturally occurring wasps of the pollinator species were collected on sticky traps or by using an aspirator as they were arriving at receptive trees. In two cases we collected wasps emerging from ripe figs by placing mesh bags around individual figs; only a single wasp per fig fruit was examined to assure independence. In all fig species except one we sampled from several independent flowering or fruiting events (crops); number of examined wasps per event: *F. max.*: 723; *F. ins.*: 311, 1117; *F. pop.*: 564, 699, 620, 988, 715, 979, 459; *F. obt.*: 107, 396, 206, 160, 241; *F. nym.*: 709, 302, 411; *F. cit.*: 621, 479, 1017, 687, 919**.** Wasps were examined under a light microscope, with the examiner blind to species when possible, to detect presence or absence of pollen grains in their pollen pockets (active pollinators) or on their body (passive pollinators) (Fig. 1.2b-d). We fitted a generalized linear model with binomial errors, a logit link, and an overdispersion parameter, using natural pollen-free (NP-) wasps (out of total number of wasps) for each crop as the response variable, and species as the explanatory variable. Results did not change if we instead used a Kruskal-Wallis test to examine whether the proportion of NP- wasps differed across species.

Phylogenetically independent contrasts

We used the PDAP module of Mesquite to calculate phylogenetically independent contrasts (Midford *et al.* 2008). Because the dependent variable is a wasp characteristic, we based calculations on the best known wasp phylogeny (Machado *et al.* 2005, Machado pers. comm.). We used molecular branch lengths; results did not change if we used equal branch lengths. We treated each host as associated with only one (the most common) wasp species. The results did not change if we instead based calculations on an alternative wasp phylogeny (Jackson *et al.* 2008) or the fig phylogeny (Jackson *et al.* 2008), using either equal or molecular branch lengths.

3. Results

Experimental investigation of fitness cost for wasps that do not pollinate

We found host sanctions against cheating (AP-) wasps in all actively pollinated fig species. These sanctions were a combination of 1) increased abortion of P- figs (M_R), and 2) reduced number of wasp offspring in P- figs that were not aborted (O_R) (Table S2, appendix chapter 1). In contrast, the passively pollinated fig species showed no evidence of sanctions: there was no difference between P- and P+ figs in either the likelihood of abortion, or the number of wasp offspring that developed from a fig (Table S2, appendix chapter 1). Specifically, the relative proportion of P- figs that matured (M_R) was significantly lower in the actively pollinated species than the passive (ANOVA $F_{5,9} = 39.5$, $p < 0.0001$; planned contrast $t_9 = -7.7$, $p < 0.0001$; Fig. 1.1a). Further, the relative number of offspring in unaborted P- figs (O_R) was significantly lower in the actively pollinated species than the passive (ANOVA $F_{5,5} = 7.7$, $p < 0.05$; planned contrast $t_5 = -4.1$, $p < 0.01$; Fig. 1.1b). The resulting relative fitness for a single foundress P- wasp (W_R) was

significantly lower in actively pollinated species than in passive (ANOVA $F_{5,8} = 70.7$, $p < 0.0001$, planned contrast $t_8 = -10.6$, $p < 0.0001$; Fig. 1.1c). There was no evidence that the experimental AP- treatment reduced the number of eggs a wasp carried or could lay (appendix chapter 1). Across all species, all figs aborted if neither a wasp nor pollen entered (Table S2, appendix chapter 1).

Sanction strength (defined as $1\text{-}W_R$ (see Wenseleers & Ratnieks 2006)) varied greatly across the actively pollinated species. The relative proportion of P- figs that matured (M_R) ranged from 2.8% (*F. citrifolia*) to 78% (*F.popenoei)* (ANOVA $F_{3,7} = 32.3$, $p < 0.001$, Tukey HSD $p < 0.05$) (Fig. 1.1a. The relative number of wasp offspring produced in un-aborted P- figs (O_R) ranged from 4.9% (*F. citrifolia*) to 88% (*F. popenoei*) of the number produced in P+ figs (ANOVA $F_{3,4} = 6.3$, $p = 0.053$**)** (Fig. 1.1b). Therefore, across the actively pollinated species, the combined effects of abortion and offspring reduction produced large differences in estimated relative fitness for P- wasps (W_R), ranging from 0.14% (*F. citrifolia*) to 67% (*F. popenoei*) (ANOVA $F_{3,7} = 55.6$, $p < 0.0001$, Tukey HSD $p < 0.05$) (Fig. 1.1c).

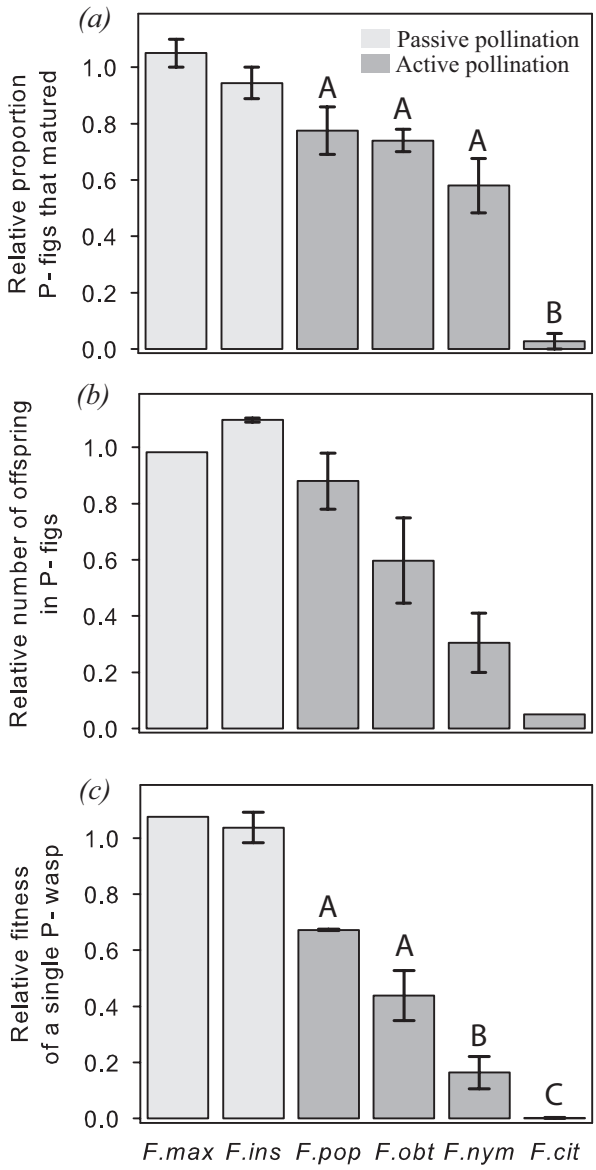

Figure 1.1. The two components of sanctions and the resulting relative fitness of a cheating wasp compared across species. *(a)* Relative proportion of figs that matured (did not abort) after experimental introductions of wasps without pollen (AP-) compared to wasps with pollen (P+) (M_R). *(b)* Relative number of wasp offspring emerging from un-aborted P- figs compared to P+ figs (O_R). *(c)* The resulting experimentally determined relative fitness of a single foundress AP- wasp compared to a P+ wasp (W_R). Letters represent significantly different subsets within the actively pollinated species. Error bars represent 1 s.e.m.

Foundress distributions

The proportion of wasps that were single foundresses varied considerably across the actively pollinated fig species: in *F. obtusifolia* (A), 71 ± 8.2 (s.e.m.) % , in *F. citrifolia* (A) 52 ± 5.3%, in *F. nymphaefolia* (B) 24 ± 10.6%, and in *F. popenoei* (B) 6.9 ± 2.2% (GLM, binomial errors, χ^2_3= 76.1, p < 0.0001; letters represent significantly different subsets). Therefore, in addition to the difference in sanction strength described above, an average P- wasp in *F. popenoei* would be 7 times less likely to experience full sanctions than an average P- wasp in *F. citrifolia*.

Field survey of natural pollen-free wasps

The proportion of natural pollen-free (NP-) wasps varied significantly across species (GLM, binomial errors, χ^2_5= 93.0, $p < 0.0001$; Fig. 2). No passively pollinating wasp (out of 2151 sampled) was caught without pollen, whereas all actively pollinating wasp species exhibited some individuals that did not carry any pollen (planned contrasts, $p<0.0001$). Within the actively pollinated species, pollen-free wasps were an order of magnitude more common in wasps associated with *F. popenoei* (5%) than in the other species (0.5%) (GLM, binomial errors, χ^2_3= 65.9, $p < 0.0001$; pairwise sequential Bonferroni corrected contrasts, $p < 0.0001$) (Fig. 1.2).

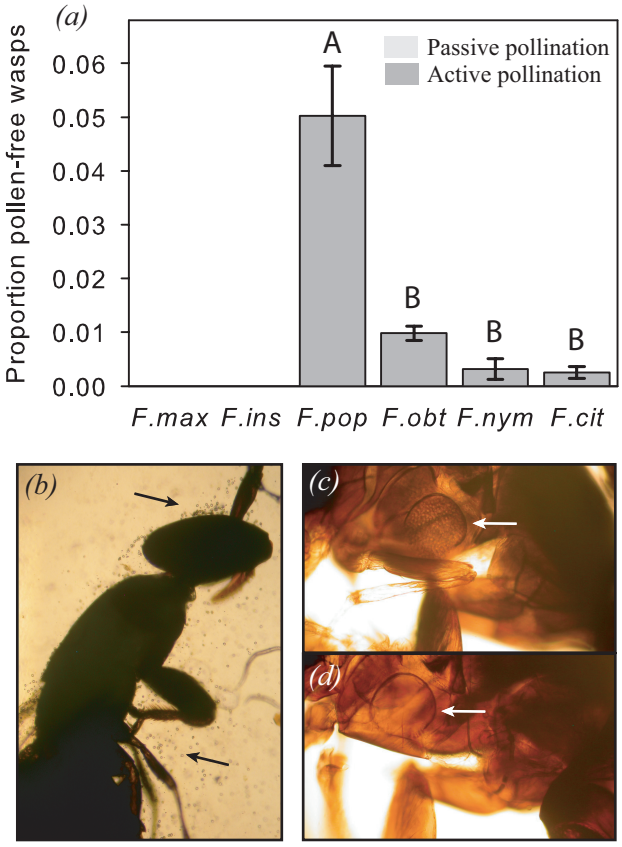

Figure 1.2. Wasps of the pollinator species do not always carry pollen. *(a)* The proportion of naturally occurring pollen-free wasps (NP-) varied across the studied fig species. NP- wasps were only found in association with actively pollinated fig species. Letters represent significantly different subsets within the actively pollinated species; error bars indicate 1 s.e.m. *(b)* Passive pollinator of *F. insipida* with pollen grains scattered all over her body. *(c, d)* Two active pollinator wasps of *F. nymphaefolia*, one with her pollen pocket full of pollen grains (arrow, *c*), the other with an empty pollen pocket (arrow, *d*). In both *(c)* and *(d)*, the wasp's head is just outside the lower right corner.

Further, across the actively pollinated species the proportion of pollen-free wasps negatively correlated with sanction strength (Pearson correlation on log transformed data, r = -0.996, p <0.01) (Fig. 1.3). This relationship persisted when we controlled for phylogenetic dependencies

(PIC, r = -0.995, p <0.01). Across the actively pollinated species studied, there was no significant relationship between the proportion pollen-free wasps and the proportion wasps that were single foundresses (Pearson correlation, r = -0.46, p = 0.54).

F. popenoei and *F. obtusifolia* each have two cryptic pollinator species (Molbo *et al.* 2003). We found pollen-free wasps in each of these pollinator species; there was no support for pollen-free wasps belonging exclusively to one of the cryptic wasp species (appendix chapter 1, Table S3). *F. citrifolia* and *F. nymphaefolia* have only one known pollinator species each (Molbo *et al.* 2003; Machado *et al.* 2005); all tested NP- and P+ wasps in *F. citrifolia* belonged to the known species.

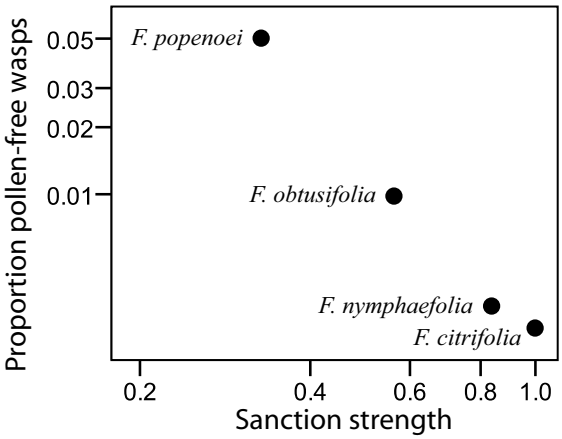

Figure 1.3. The proportion of naturally occurring pollen-free wasps was negatively correlated with sanction strength (1 - W_R) across actively pollinated fig species.

4. Discussion

This study provides three novel findings relevant to mutualism stability. First, we show that host sanctions against non-cooperative symbionts vary dramatically in form and intensity across fig

species. Second, we document the existence of pollen-free individuals ("cheaters") within the otherwise mutualistic pollinator wasp species. Third, across the actively pollinated fig species, we show that the proportion of pollen-free wasps is negatively correlated with sanction strength. Finally, we combine the results from our study with previous fig studies to give a phylogenetic overview of our current knowledge of host sanctions and wasp cheating in the fig tree – fig wasp mutualism. Together, these studies demonstrate that the form and strength of sanctions in the host, and the corresponding characteristics of the pollinators vary greatly across the fig tree – fig wasp mutualism.

Host sanctions in figs

Results from previous pollen exclusion experiments in figs show or suggest lower offspring numbers for wasps that did not pollinate, and/or increased abortion of figs that received wasp eggs but no pollen (Galil & Eisikowitch 1971; Nefdt 1989; Jousselin & Kjellberg 2001; Jousselin *et al.* 2003a; Tarachai *et al.* 2008). Host sanctions have been detected in fig species representing all major subgenera of *Ficus* (see the phylogenetic overview in Fig. 1.4). However, the previous studies only examined actively pollinated species, with little or no replication either of species, or of trees within species (Fig 1.4; summary in Herre *et al.* 2008). Our study design allows us to directly compare sanction strength both across several closely related actively pollinated fig species, and across distantly related groups of figs that represent different pollination syndromes. In the actively pollinated species, the sanction strengths we measured here (0.33 to 0.999 in *F. popenoei* and *F. citrifolia* respectively) bracket those reported previously (0.34 to 0.80 in *F. burtt-davyii* and *F. montana* respectively) (Fig. 1.4).

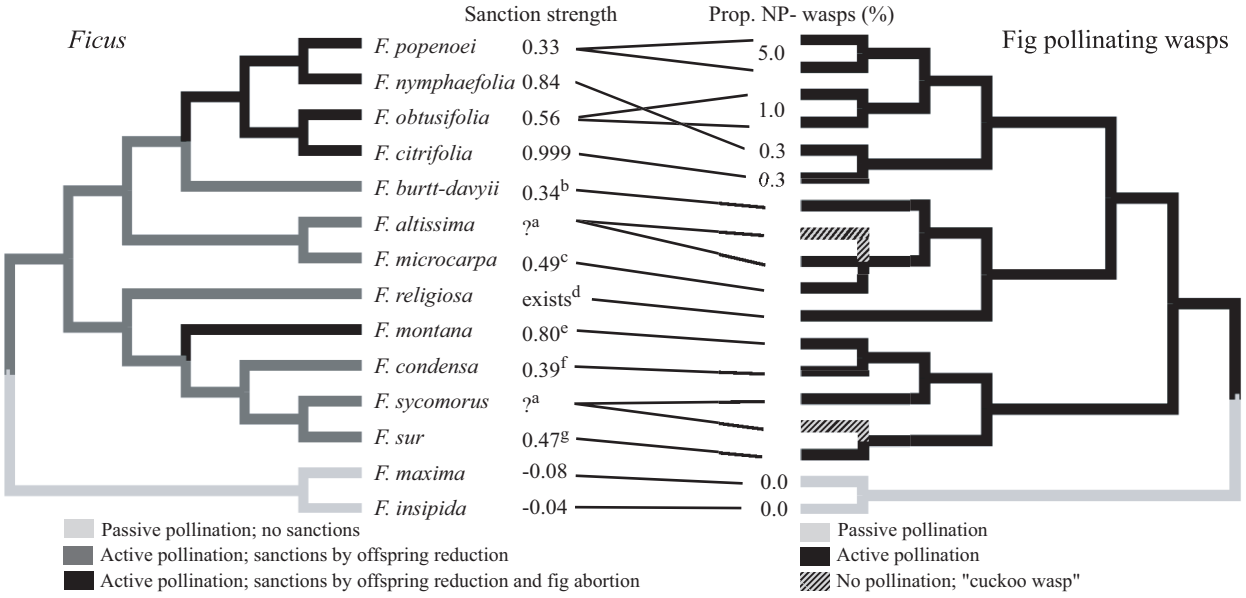

Figure 1.4. Phylogenetic relationship between the fig species that have been studied for sanctions and their pollinating wasps. Because the sanction strength value does not take into account the likelihood of being a single foundress in the respective species, effective sanction strength is likely to be lower in species with multiple foundresses. The proportion of naturally occurring pollen-free wasps (NP-) in the pollinating species is indicated where known. Phylogenetic relationships based on (Machado *et al.* 2001; Machado *et al.* 2005; Rønsted *et al.* 2005; Jackson *et al.* 2008, Machado pers. comm.). Placement of wasps associated with *F. altissima* and *F. religiosa* are inferred from the wasp taxonomy. [a]*F. altissima* and *F. sycomorus* are associated with "cuckoo" wasp species that have lost their active pollination behaviour; we hypothesize that they have weak, if any, sanctions. In earlier pollen-free experiments, figs are monoecious and the fitness reduction significant unless otherwise noted; sample sizes [P+,P-] and relevant notes: [b][6,12] (Nefdt 1989); [c][12,11], $p = 0.07$ (Jousselin *et al.* 2003a); [d]no data or statistical tests presented (Galil & Eisikowitch 1971); [e][60,60], dioecious (Tarachai *et al.* 2008); [f][16,16], dioecious (Jousselin & Kjellberg 2001); [g][4,3], experimental figs had two foundresses (Nefdt 1989).

The passively pollinated species in our study represent the most basal lineage of *Ficus*, and in contrast with previously studied fig species (Herre *et al.* 2008, Fig. 1.4), there was no indication of host sanctions in these species. Further, we found no evidence of pollen-free individuals in the associated wasp species. Passively pollinating wasps do not actively expend energy pollinating, and they cannot easily avoid carrying pollen. We suggest that although passively pollinated fig

species invest more in pollen production, they benefit from a low incidence of pollen-free wasps, which makes sanction mechanisms unnecessary. In contrast, actively pollinating wasps actively expend time and energy on pollination, and omitting even one of a chain of required pollination behaviours would prevent a fig from being pollinated. Therefore, this behaviour could easily be lost, and there may be wasp incentives to do so. Although actively pollinated fig species benefit from considerable lower costs of pollen production (Kjellberg *et al.* 2001), all existing studies of actively pollinated fig species suggest that they need effective sanction mechanisms in order to maintain highly cooperative pollinators (Fig. 1.4; Herre *et al.* 2008; this study).

The existence of "cheaters" within the mutualistic wasp species

In the fig tree – fig wasp system there are two well-known groups of non-mutualistic wasp species: 1) numerous taxa of parasitic wasps that mostly oviposit from the outside of figs and do not pollinate (West *et al.* 1996; Rasplus *et al.* 1998; Kerdelhue *et al.* 2000; but see Jousselin *et al.* 2001), and 2) two species of wasps from lineages of active pollinators that have lost their ability to pollinate, and have become parasites or "cuckoos" (Galil & Eisikowitch 1968; Compton *et al.* 1991; Peng *et al.* 2008; Fig. 1.4). In contrast, we here report for the first time the existence of pollen-free, "cheating", wasp individuals that belong to otherwise mutualistic pollinating wasp species, and not to separate, parasitic species. Since these wasps will be unable to pollinate but still will be able to lay eggs, they are effectively cheaters with respect to the tree's seed production (female function). If the tendency to not collect pollen is heritable, then such wasps are also detrimental to the tree's male function because their daughters are not likely to disperse pollen.

We found pollen-free individuals in natural populations of all actively pollinating wasp species, including all known cryptic wasp species of *F. popenoei* and *F. obtusifolia*. Interestingly, although the cryptic species in *F. obtusifolia* appear to be sister species, those in *F. popenoei* are not (Molbo *et al.* 2003; Jackson *et al.* 2008), suggesting that the high levels of pollen-free wasps found in the two cryptic species associated with *F. popenoei* may represent two independent evolutionary events. It is currently unclear if the pollen-free wasps derive any benefit, and if so how large, from not carrying pollen. Potential benefits include energy savings from not carrying the pollen weight, and time savings from not collecting and depositing pollen.

The association between sanction strength and the prevalence of pollen-free wasps

In systems with repeated interactions between individuals, direct punishment of uncooperative individuals is known to induce future cooperative behaviour. For example, in the reef fish – cleaner fish mutualism, host punishment of cheating cleaners increased cooperation levels in future interactions with the same host individual (Bshary & Grutter 2002; Bshary & Grutter 2005). Many relationships, however, are not characterized by repeated interactions between the punisher and the punished. Recent studies of intraspecific systems lacking repeated interactions suggest that the level of cheating in a population will negatively correlate with the *expected* level of punishment. In social insects, reproductive workers (cheaters) are rarer in species where the probability of nestmates killing worker-laid eggs is higher (Wenseleers & Ratnieks 2006), and across human societies, the tendency for cooperation in economic games is positively correlated with the tendency to punish uncooperative individuals (Henrich *et al.* 2006).

Here we have shown a similar pattern in a mutualism, where the interacting individuals belong to different species and do not interact repeatedly. Across the actively pollinated fig species in our study, the prevalence of naturally pollen-free (NP-) wasps was negatively correlated with host sanction strength, and this relationship persisted when we controlled for phylogenetic dependencies. Although data for the prevalence of NP- wasps is currently unavailable for the previously studied fig - wasp pairs (Fig. 1.4), we expect NP- wasps to be relatively more common in fig species where sanctions are weak. We would similarly expect the fig species associated with the "cuckoo" wasps (*Ficus sycomorus* and *F. altissima*) to have relatively weak sanctions (Herre *et al.* 2008, Fig. 1.4).

The fig sanctions described in this study are likely to be a modification of the universal plant trait of aborting unpollinated flowers. Fig trees, too, abort figs that are both unpollinated and unoviposited (this study and (Bronstein 1988), also see (Herre 1989)). However, as shown in this study and others (Galil & Eisikowitch 1971; Nefdt 1989; Jousselin & Kjellberg 2001; Jousselin *et al.* 2003a; Tarachai *et al.* 2008), fig trees often retain unpollinated figs in which wasps have oviposited. We note that in monoecious species, a seedless fig can still contribute to a fig tree's fitness if at least some of the offspring wasps disperse pollen from their natal fig. While we suspect that the immediate reason for trees to apply "sanctions" is likely to direct resources to those figs that are the most profitable (most seeds and wasps per tree investment), such sanctions would also restrain the spread of the pollen-free trait in the wasp populations if the pollen-free trait is heritable. In contrast, wasps should be selected to increase the likelihood that oviposited flowers will be provisioned, and reduce the likelihood of fig abortion, regardless of pollination status. We suspect that whether individual flowers are provisioned or entire figs are aborted will

be determined from the chemical/physical interaction between the fig inflorescences and some combination of pollination and the liquid deposited by the wasps during oviposition (Verkerke 1989).

The relationship between the spatial precision of sanctions and the spatial distribution of symbionts will be important in determining the effectiveness of sanctions in any mutualism where multiple symbionts interact simultaneously with a host (see also (Denison 2000; Bever *et al.* 2009). For example, if sanctions operate on the fig level, pollen-free wasp might largely evade sanctions in fig species that routinely have multiple foundresses (such as *F. popenoei* and *F. sycomorus* (Herre 1989; Compton *et al.* 1991)) by free-riding on the pollination efforts of other foundresses. Alternatively, if sanctions operate on the level of individual flowers within figs, pollen-free wasps would be relatively more exposed to sanctions even in fig species with many foundresses. We found a negative but non-significant relationship between the likelihood of wasps being single foundresses and the proportion pollen-free wasps, the direction being consistent with sanctions acting on the fig level. Further studies of the figs are needed to identify the level of precision and mechanism of sanctions, and to attempt to quantify the relative costs of sanctions across species. Further studies of the wasps are needed to determine if naturally occurring pollen-free wasps inherit this trait from their mothers, and whether any fitness benefits of the pollen-free trait are large enough to explain its persistence despite the sanctions.

In conclusion, we found host sanctions in all actively pollinated fig species, but not in passively pollinated fig species. Further, we found pollen-free individuals in all species where wasps easily can cheat by omitting any of the time consuming behaviours associated with active pollination.

Within these actively pollinated fig species, pollen-free wasps were most common in the species with the weakest sanctions. Combined with previous studies, our results indicate that the mechanisms that maintain mutualism stability are not fixed in form or intensity, and that within the fig tree – fig wasp mutualism they have changed dramatically over the course of 80 MYA of co-adaptation. Such change in host sanction and symbiont response is likely to be a more general property across other mutualisms, analogous to "arms races" in overtly antagonistic interactions.

APPENDIX CHAPTER 1

Materials and Methods

Table S1. Basic information on the studied fig species

Section	**Fig species**	**Wasp pollinator** [a,b]	**Mean number of female flowers** [c, d]	**Mean foundress number** [e]
Pharmacosycea *Passively pollinated*	*Ficus maxima*	*Tetrapus americanus*	445	1.5
	F. insipida	*T. costaricanus*	1230	3.6
Americana *Actively pollinated*	*F. popenoei*	*Pegoscapus gemellus A* *P. gemellus B*	1124	4.1
	F. obtusifolia	*P. hoffmeyeri A* *P. hoffmeyeri B*	976	1.1
	F. nymphaefolia	*P. piceipes*	675	1.9
	F. citrifolia	*P. tonduzi*	325	1.4

[a] (Wiebes 1995)
[b] (Molbo *et al.* 2003)
[c] (Kjellberg *et al.* 2001)
[d] (Herre 1989)
[e] Based on data in this paper; see also Herre 1989.

Results

Table S2. Proportion aborted figs and number of mature wasp offspring in un-aborted figs after experimental introductions of wasps with pollen (P+) or without pollen (P-). All figs aborted when neither wasp nor pollen entered ("no foundress"; F-). N indicates the number of individual figs in each treatment. Fisher's exact test (*), student's t-test (unmarked) or separate variances t-test (†) were used for statistical analyses. Bold species names indicate passively pollinated species.

	F-	P+		P-			P+		P-		
Species; tree #	N	Prop. abort.	N	Prop. abort.	N	*p* *	Mean offspring (s.e.m.)	N	Mean offspring (s.e.m.)	N	*p*
F. maxima; 1	27	0.087	23	0	20	0.49	207.6 (4.7)	21	204.0 (11.3)	20	0.77†
F. maxima; 2	-	0	9	0	7	1					
F. insipida; 1	-	0	12	0	12	1	160.8 (11.2)	12	175.6 (12.7)	12	0.39
F. insipida; 2	2	0.25	4	0.33	6	1	119.0 (41.3)	3	131.5 (23.9)	4	0.79
F. popenoei; 1	45	0	43	0.135	37	<0.05	160.3 (4.2)	12	124.3 (7.4)	32	<0.001†
F. popenoei; 2	10	0.036	55	0.333	78	<0.0001	136.5 (6.5)	15	133.1 (5.6)	23	0.70
F. obtusifolia; 1	4	0	25	0.217	23	<0.05	265.2 (20.1)	13	118.1 (19.2)	10	<0.0001
F. obtusifolia; 2	10	0	35	0.296	54	<0.0001	221.3 (14.0)	21	165.7 (12.8)	34	<0.01
F.nymphaefolia;1	9	0.035	29	0.294	34	<0.01	157.3 (9.9)	15	15.8 (1.2)	24	<0.0001†
F.nymphaefolia;2	8	0.083	25	0.44	25	<0.01	223.8 (5.4)	15	99.7 (6.0)	14	<0.0001
F.nymphaefolia;3	14	0	7	0.6	5	<0.05	167.3 (22.2)	6	61.0 (24.0)	2	<0.05
F. citrifolia; 1	46	0.063	16	1	16	<0.0001					
F. citrifolia; 2	4	0	5	1	6	<0.01					
F. citrifolia; 3	35	0.242	33	1	32	<0.0001					
F. citrifolia; 4	18	0.088	34	0.897	29	<0.0001	122.7 (11.8)	10	6.0 (0.58)	3	<0.001

1. Validation of the experimental method

In an additional experiment, we tested whether the experimental no-pollen treatment (opening and manipulating the fig before females had emerged from their galls) impaired the wasps' initial egg numbers or subsequent ability to lay eggs. If so, that could potentially explain why AP- wasps had fewer offspring than P+ wasps in our experiments.

We produced AP- and P+ wasps of *P. tonduzi*, pollinator of *F. citrifolia*, as described in the methods section, from 10 different figs in each treatment. Some wasps from each fig were gently pressed on microscope slides, and their eggs were counted (Nefdt & Compton 1996). Other wasps from the same figs were introduced as single foundresses into receptive figs on trees, and were left undisturbed to oviposit. Figs were collected after 36-48 hours, when wasps were dead, and the number of eggs remaining inside each wasp was counted. Fig wasps emerge with a full set of eggs, and do not produce more over their lifespan (Copland & King 1973).

Before oviposition, there were slightly more eggs in AP- wasps than in P+ wasps (t-test, t_{81} = 2.02, $p < 0.05$) (Fig. S1). After oviposition, there was no statistical difference between the two groups (Mann-Whitney U-test, Z= - 1.68, $p = 0.09$), although the mean for the AP- group was slightly higher than in the P+ group (Fig. S1). Therefore, there was no indication that the experimental no-pollen treatment impaired the AP- wasps' initial egg number or the number of eggs they laid.

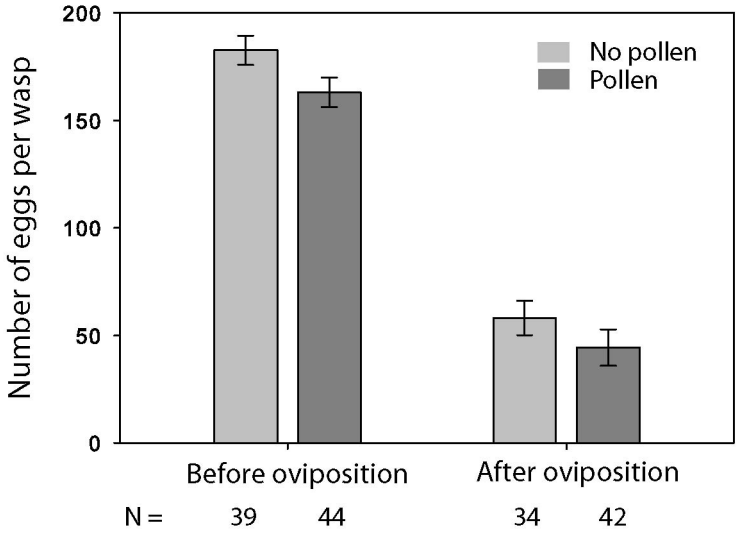

Figure S1. Average number of eggs per wasp in AP- (no pollen) or P+ (pollen) treatment groups before and after oviposition. The number of wasps examined in each group is indicated below each bar. Error bars indicate 1 s.e.m.

2. Do pollen-carrying and pollen-free wasps belong to different cryptic species?

F. obtusifolia and *F. popenoei* have two pollinator species each. These wasp species are morphologically very similar, but can be identified to species using molecular methods. We wanted to know whether the pollen-free wasps belonged exclusively to one of the two cryptic species, or if they were present in both.

Naturally occurring unmanipulated wasps of the pollinator species were collected as they arrived at receptive trees and were examined for presence (P+) or absence (NP-) of pollen; the majority were P+. For a subset of trees, all collected NP- wasps, and an equal or larger number of P+ wasps, were identified to species by sequencing the mitochondrial COI (cytochrome oxidase subunit I) gene (Molbo *et al.* 2003).

Pollen-free wasps existed in all known wasp species - there was no support for NP- wasps belonging exclusively to one of the cryptic wasp species (table S3).

Table S3. Distribution of NP- and P+ wasps in the cryptic wasp species of *F. popenoei* and *F.obtusifolia*. The number of independent flowering events sampled is given after each fig species' name.

Fig species	Wasp type	Number of individual wasps	
F. popenoei (3)		*P. gemellus A*	*P. gemellus B*
	NP-	8	77
	P+	10	75
F. obtusifolia (2)		*P. hoffmeyeri A*	*P. hoffmeyeri B*
	NP-	2	2
	P+	8	6
F. citrifolia (1)		*P. tonduzi*	
	NP-	4	
	P+	9	

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CHAPTER 2

PRECISION OF HOST SANCTIONS IN THE FIG TREE – FIG WASP MUTUALISM: CONSEQUENCES FOR UNCOOPERATIVE SYMBIONTS

Abstract

The persistence of mutualisms in the face of cheating has long been a conceptually and empirically challenging problem. Host sanctions have been shown to reduce the fitness of uncooperative symbionts, and therefore may contribute to mutualism maintenance. However, if multiple symbionts interact with each host or host module, sanctions may not be precise enough to effectively punish cheating symbionts individually. Such lack of precision could greatly reduce the effectiveness of sanctions. Here we report the level of precision of host sanctions in the mutualism between fig trees and their pollinating wasps. In this system, trees can reduce the fitness of non-pollinating wasps by aborting figs or reducing wasp offspring numbers. Using a combination of field experiments and molecular parentage analyses, we show that sanctions in *Ficus nymphaefolia* (pollinator *Pegoscapus piceipes*) act at the level of entire figs, not at the level of individual flowers within a fig. In experimental fig fruits with one pollinating and one pollen-free "cheating" wasp, the two mothers produced on average equal numbers of offspring. Such modular sanctions allow pollen-free wasps to free-ride in figs with multiple foundresses.

1. Introduction

Mutualisms, relationships between two species where both partners benefit, are of fundamental ecological importance. For example, many trees depend on mycorrhizal fungi to access nutrients, legumes and other plants benefit from nitrogen-fixing soil bacteria, and many animals, including humans, rely on gut microbes to help absorb otherwise inaccessible nutrients (Herre *et al.* 1999; Bäckhed *et al.* 2005). Despite their importance, the evolution and maintenance of mutualisms are not well understood, particularly when cooperation is costly and not merely a by-product (Trivers 1971; Axelrod and Hamilton 1981; Bull and Rice 1991; Herre *et al.* 1999; Sachs *et al.* 2004; West *et al.* 2007). What prevents beneficial symbionts from turning into parasites? Natural variation in partner cooperation levels has been shown in many different mutualisms (Compton *et al.* 1991; Wolters *et al.* 1997; Pellmyr and Leebens-Mack 2000; Simms *et al.* 2006; Peng *et al.* 2008; Bever *et al.* 2009; Jandér and Herre 2010; Sachs *et al.* 2010). Individuals that reap the benefits from an interaction without paying the costs would gain a relative fitness advantage, increase in frequency, and over time break down the mutualism. Therefore, mutualisms with costly cooperation likely require mechanisms that limit cheating (Axelrod and Hamilton 1981).

One mechanism that might limit cheating in mutualisms is host sanctioning, which has now been documented in several plant systems (yucca – yucca moths, figs – fig wasps, legumes- rhizobia, allium-mycorrhizae, and *Glochidion* – epicephala moths) (Pellmyr and Huth 1994; Jousselin *et al.* 2003; Kiers *et al.* 2003; Simms *et al.* 2006; Tarachai *et al.* 2008; Bever *et al.* 2009; Goto *et al.* 2010; Jandér and Herre 2010). Although the exact mechanisms are still unclear, host resources seem to be selectively allocated to the symbiont(s) that most benefit the host, which in turn increases the fitness of cooperative symbionts. For example, yuccas and *Glochidion* selectively

abort fruits that are overexploited by their pollinating moths, and legumes selectively withhold resources from those nodules that contain the least productive nitrogen-fixing bacteria (Pellmyr and Huth 1994; Kiers *et al.* 2003; Simms *et al.* 2006; Goto *et al.* 2010). Fig trees can abort or reduce wasp offspring numbers in fig fruits where fig wasps have oviposited but not pollinated (Jousselin *et al.* 2003; Herre *et al.* 2008; Tarachai *et al.* 2008; Jandér and Herre 2010).

Host sanctions have the potential to reduce the fitness of uncooperative symbionts, and thereby prevent their spread through the population. Nevertheless, an important aspect in the documentation of sanctions is missing. So far, sanctions have been documented on the level of easily observable host modules such as individual fruits or individual nodules; we can call this modular sanctions (Weyl *et al.* 2011). However, a crucial factor in determining the effectiveness of sanctions is whether sanctions can actually pinpoint cheating individuals (or individual bacterial lineages) or if they imprecisely reduce the fitness of several individuals as a group. If, for example, multiple symbionts occupy a host module, and sanctions act at the modular level, then rare cheating symbionts might partly avoid sanctions by free-riding on the good behaviour of other symbionts within the module (Fig 2.1b) (Denison 2000; Simms and Taylor 2002; Jandér and Herre 2010). When multiple symbionts occupy a host module, sanctions that can target individual symbionts within a host module (we call this individualized sanctions) will be more effective (Fig 2.1b). For example, a recent model of the legume – rhizobia mutualism showed that when mixed infections occurred within a nodule, hypothetical sanctions that could target individual rhizobial lineages within a nodule (individualized sanctions) always selected for higher levels of rhizobial cooperation (Friesen and Mathias 2010). In the simple case of each

host module interacting with only one symbiont, modular sanctions will always equal individualized sanctions (Fig 2.1a).

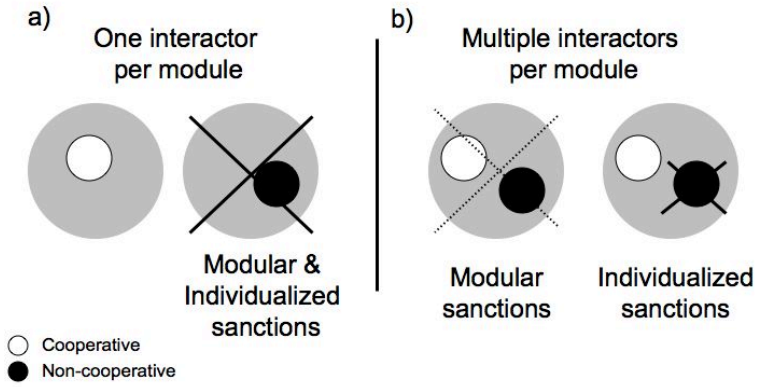

Figure 2.1. Large grey circles represent host modules, smaller circles represent cooperative (white) or non-cooperative (black) symbiont individuals or strains interacting with each module. The X represents reduction in resources due to host sanctions. (a) When there is only one interactor per module, modular sanctions will always also be individualized sanctions. (b) When there are multiple interactors per module, sanctions can be modular, or individualized.

Distinguishing between modular sanctions and individualized sanctions is important in mutualisms where multiple symbionts interact with each host module (eg. fruit, nodule), a rather common situation. In the majority of fig species, individual figs routinely have more than one female wasp enter each fig fruit (Herre 1989; also inferred from flower numbers in Kjellberg *et al.* 2001), and individual flowers/fruits of yuccas and *Glochidion* commonly experience oviposition by more than one moth (Pellmyr and Leebens-Mack 2000; Kato *et al.* 2003). In legumes, mixed infections (more than one strain per nodule) occur in 7-74% of nodules under laboratory conditions (Denison 2000; Gage 2002; Sachs *et al.* 2010). Therefore, the question of

the precision level of sanctions is relevant for many well-studied mutualisms, and probably also for many less-studied mutualisms.

We investigated the precision of sanctions in the mutualism between fig trees and their pollinating fig wasps, in which both partners are dependent on the other for reproductive success. This is a particularly powerful study system because lifetime reproductive success can easily be quantified for the wasps, and wasp cooperation levels can be manipulated (Herre 1989; Jandér and Herre 2010). The number of interacting symbionts (wasps) per module (fig) is also easily quantified (Herre 1989) and manipulated. Fig trees are pollinated by species-specific fig wasps, and fig wasp larvae develop inside figs. Fig trees produce hundreds of tiny flowers inside each hollow syconium (hereafter called fig). The minute fig wasps are attracted by the scent of the flowering fig; one or several females (foundresses) squeeze inside the fig through a narrow opening, pollinate and lay their eggs in some of the flowers, and then die inside the fig. In the monoecious fig species that we study here, both wasps and seeds are produced in the same figs, and pollination is active: wasps actively collect pollen, store it in specialized pockets, and deposit it using their front legs (Frank 1984; Jandér and Herre 2010). Wasps typically pollinate each flower in which they have laid an egg, but also unoviposited flowers (Jandér unpublished data). A flower that receives an egg (with or without pollen) will develop into a gall containing a wasp, whereas a flower that only receives pollen will develop into a seed. Upon maturation, female wasps mate, emerge from their galls, actively gather pollen from the stamens inside their natal fig, then emerge from the fig and fly off in search of a flowering fig on a different tree.

Fig wasps of the actively pollinating species typically carry pollen (P+), but a small fraction of individuals in natural populations do not carry pollen (NP-) (Jandér and Herre 2010). Fig wasps can be manipulated to produce artificially pollen-free wasps (AP-) (Jousselin *et al.* 2003; Jandér and Herre 2010). Our previous experiments using individual foundress AP- wasps showed that fig trees are able to impose sanctions that lower the fitness of wasps that do not pollinate. Unpollinated figs (with wasp eggs) are more likely to abort than are pollinated figs, and in figs that mature, fewer wasp offspring emerge from unpollinated than from pollinated figs (Jandér and Herre 2010). We term this latter type of sanction "offspring reduction". Both sanction strength and the prevalence of naturally occurring pollen-free wasps (NP-) vary across species, such that NP- wasps are rare in host species with severe sanctions and most common in the species with the weakest sanctions (Jandér and Herre 2010). It is currently not known if NP- wasps derive any benefits from not carrying pollen. Potential benefits include escaping ants and damage from crowding males by emerging earlier from the fig, energy savings from not carrying pollen in flight, and time savings from not depositing pollen (Jandér MS).

The level of precision of sanctions by fig trees is important in determining how effectively sanctions might prevent the spread of NP- wasps in the respective populations. For example, in *F. popenoei*, a fig species associated with a relatively high prevalence of NP- wasps (5%), 93% of wasps shared their fig with one or more (up to 20) additional foundresses (Jandér and Herre 2010). If sanctions target the entire fig fruit (modular sanctions), pollen-free wasps could free-ride on the pollination efforts of other foundresses with whom they share a fig. On the other hand, if sanctions target individual flowers within the fig (individualized sanctions), pollen-free wasps could not evade sanctions by sharing a fig with others. Of the two different types of host

sanctions in *Ficus* – fig abortion and reduction in wasp offspring – only offspring reduction has the potential to sanction individual flowers and thus individual wasps. Offspring reduction is a significant component of sanctions. On average across the four studied Panamanian species, offspring reduction is responsible for 52% of the total fitness reduction for wasps that do not pollinate (eg. 35% in *F. popenoei*, 62% in *F. nymphaefolia*) (Jandér and Herre 2010). In the eight non-Panamanian fig species for which data are available, offspring reduction is responsible for 100% of the total fitness reduction in seven fig species, and 45% in the eighth species (Tarachai *et al.* 2008; Jandér and Herre 2010).

Although it would be ideal to know the level of precision of sanctions in *F. popenoei* where NP-wasps are so common, for practical reasons we chose to study a closely related fig species, *F. nymphaefolia* (Machado *et al.* 2005; Jackson *et al.* 2008). In contrast to *F. popenoei, F. nymphaefolia* has 1) a sufficiently high proportion of single-foundress figs to produce the P+ and AP- wasps that we need for the introduction experiments (*F. nymphaefolia* 41%, *F. popenoei* 17%), and 2) a sufficiently strong reduction in wasp offspring numbers in unpollinated figs for us to detect a difference between the P+ and AP- foundress when introduced into the same fig, should sanctions be on the flower level (offspring numbers of AP- foundresses relative to that of P+ foundresses when introduced singly into figs were 30% in *F. nymphaefolia* and 88% in *F. popenoei*) (Jandér and Herre 2010). Under natural conditions, *F. nymphaefolia* has two or more foundresses per fig 59% of the time, so experimental introductions of two foundresses is realistic (Jandér and Herre 2010).

We assessed the precision of fig sanctions by setting up field experiments on two separate trees of *F. nymphaefolia* in 2008. We introduced either two P- wasps (AP- wasps, but hereafter called P-), one P- and one P+, or two P+ wasps, into each receptive fig (Fig. 2.2), then counted the number of offspring produced in each fig. We used molecular methods to partition wasp offspring from the P-P+ figs into their maternal lineages, in order to estimate the proportion of the offspring that originated from the P- and P+ foundresses respectively.

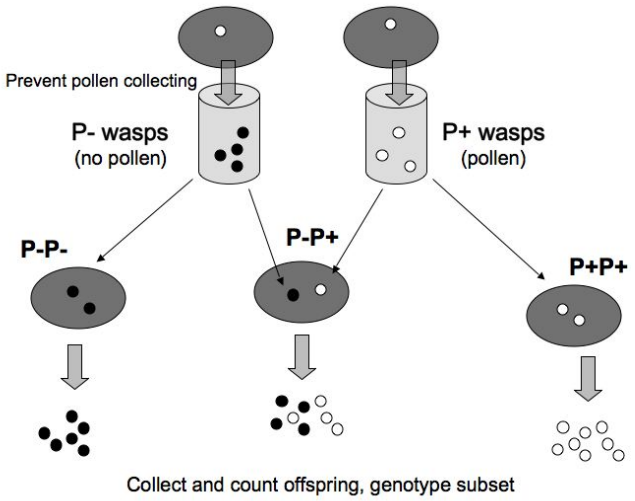

Figure 2.2. Diagram of the experimental setup. Wasp lineages originated from single-foundress figs, were subjected to either the pollen-free (AP-) or control (P+) treatment, and transferred to vials. Two foundresses were introduced into each experimental fig, and the resulting offspring collected when figs had matured.

With these data, we could distinguish between several possible precision scenarios. First we evaluated whether trees sanction entire figs, or if they can sanction individual flowers within a fig. If a tree sanctions entire figs, then, averaged across many figs, the two foundresses in P-P+ figs should produce equal number of offspring, whereas if the tree can sanction wasp larvae in

individual flowers, the P- foundress should on average have fewer mature offspring than the P+ foundress. We would not expect experimental AP- wasps such as used here to have any noticeable fitness advantage over P+ wasps. Secondly, we compared the offspring production of P- and P+ females in the P-P+ fig with half of that observed in P-P- or P+P+ figs to distinguish among four hypothesized sanction scenarios:

1A. Sanctions act on the fig level and pollination by one wasp ensures sufficient resources for all developing wasp larvae in the fig and thereby prevents sanctions.

1B. Sanctions act on the fig level but resource allocation increases with pollination level.

2A. Sanctions act on the level of individual flowers but do not sharply distinguish pollinated and unpollinated flowers – benefits from pollinated flowers "leak" to nearby flowers.

2B. Sanctions act on the individual flower level only. From a sanctions perspective, this could be the case if resources are strictly allocated to pollinated flowers only. From a different perspective, this pattern would also be seen if fertilized flowers (endosperm) provide better larval nourishment than unfertilized flowers (Verkerke 1989).

The four hypotheses generate distinct sets of predictions, which are detailed in Table 2.1. For example, under hypothesis 1A, averaged over many figs, P- and P+ wasps would produce equal number of offspring in the P-P+ fig, but on average, a P- wasp in a P-P+ fig would produce more offspring than a P- wasp in a P-P- fig (assuming that a P- wasp in a P-P- fig produces half the offspring of that fig). The same hypothesis further predicts that, on average, a P+ wasp in a P-P+ fig would produce just as many offspring as a P+ wasp in a P+P+ fig (assuming that both wasps in that fig produce equal numbers of offspring).

Table 2.1. Predictions based on the four hypotheses regarding the precision of sanctions.

	Predictions		
Hypothesis	**P-** in **P-P+** figs	Within **P-P+** figs	**P+** in **P-P+**figs
1A Fig level; 1P+ is sufficient	> ½ P-P-	**P-** = **P+**	= ½ P+P+
1B Fig level; pollen-dependent	> ½ P-P-	**P-** = **P+**	< ½ P+P+
2A Flower & nearby level	> ½ P-P-	**P-** < **P+**	< ½ P+P+
2B Flower level only	= ½ P-P-	**P-** < **P+**	= ½ P+P+

2. Methods

Field experiments

The studied trees and wasps belong to natural populations in the Barro Colorado Nature Monument, near the Panama Canal, Panama. *Ficus nymphaefolia* is here pollinated only by *Pegoscapus piceipes,* an active pollinator (Wiebes 1995; Molbo *et al.* 2003).

Following the methods described in Jandér & Herre (2010), we matched pairs of pre-receptive and mature fig trees, prevented uncontrolled pollination of the pre-receptive experimental figs by enclosing branches in mesh bags, and obtained experimental wasps from mature figs that we collected before the females had emerged from their galls. We created P- females by removing the male flowers (containing stamens) from half of these; the other half of the figs produced P+ females, which were allowed to collect pollen naturally. The experimental wasps emerging from a fig were collected into a single vial. After all wasps had emerged, we searched each empty fig for the old bodies of wasp foundresses to ensure that experimental wasps originated from figs with a single foundress. Thus, each vial of experimental wasps, either P+ or P-, represented a single maternal lineage, which greatly facilitated the later parentage analyses.

When experimental figs became receptive, each was randomly assigned to one of three wasp treatments (figure 2.2): (1) two P- wasps, (2) one P- and one P+ wasp, or (3) two P+ wasps. All experimental figs were standardized for size. The second wasp was introduced 5-10 minutes later, after the first one had completely disappeared through the ostiole. In the P-P+ figs, we alternated which type of wasp was the first to enter. Each vial of experimental wasps was used for setting up a maximum of two figs of each type, and the remaining wasps (sisters of experimental foundresses) were stored in 70% ethanol. After wasp introductions, we rebagged the branches and monitored the experimental figs regularly for abortion. We replicated the experiment on two different trees, separated in time by a week, and in space by about one kilometre; one of the trees (BCI#1) had only a small number of reachable figs, causing the sample size to be small on that tree.

At the end of the experiment, we collected experimental figs as they matured and allowed wasps to emerge within enclosed Petri dishes. We immediately placed a subset of male and female wasp offspring in ethanol, and then froze the rest of the fig contents for later dissections to determine the total number and sex of wasps emerging from each fig and the number of seeds. In three cases, wasps had already started to leave the fig at the time of collection, so we counted the number of empty wasp galls to quantify the total number of wasp offspring, but we were unable to determine either the sex or maternal lineage of the offspring that had escaped.

Very few experimental figs aborted; only figs that matured are included in this study. Figs that were lost to herbivory, or where the treatment failed (eg. due to incorrect number of foundresses, or to the occurrence of seeds in a P-P- fig) were excluded from the study. On tree BCI#1, sample

sizes were P-P-: 3, P-P+: 5, P+P+: 4; on tree BS#1 sample sizes were P-P-: 21, P-P+: 23, P+P+: 17. For each of the 28 figs within the P-P+ treatment, we aimed to genotype 20 female offspring and 20 male offspring. In a few cases we could not get the full 20 + 20 offspring, either because wasps had emerged early (three figs), or because there were fewer than 20 males in a fig (seven figs).

Molecular sorting of offspring into maternal lineages

We first sequenced 886 basepairs of the COI (cytochrome oxidase, subunit I) mitochondrial gene on sisters of the experimental foundresses to identify the mitochondrial haplotype of the maternal lineage in each vial. We extracted DNA from individual wasps using the Puregene® DNA isolation kit (Gentra), with the modified *Drosophila* protocol described in (Molbo *et al.* 2002). We used the previously designed primer pair Jerry (5'-CAA CAT TTA TTT TGA TTT TTT GG-3') (Simon *et al.* 1994) and Georgina (5'-CGD GGT ATH CCD GCT AAW CCT A-3') (Machado, C. pers. com.), product size 493 basepairs, and also developed a new primer pair Alphonse (5'-TGG GTG CTG TTT ATG CAA TTT-3') and Maisy (5'-AAA ACC TCC ACC AGG AAC AG-3'), product size 432 basepairs, overlapping 133 bp with the previous product. The PCR mix consisted of 5.6µl DNAse-free H_2O, 1µl 10x buffer, 0.5µl dNTPs, 0.3µl 50 mM $MgCl_2$, 1.25µl BSA, 0.125µl 10µM forward primer, 0.125µl 10µM reverse primer, 0.1µl Platinum® Taq DNA polymerase, and 1µl DNA, for a total of 10µl per sample. The PCR protocol for Jerry-Georgina was 94 °C for 2 min, 10 cycles of denaturation (94 °C, 30 s), annealing (46 °C, 30 sec), and extension (72 °C, 30 s), then 30 cycles of denaturation (94 °C, 30 s), annealing (48 °C, 30 sec), and extension (72 °C, 30 s), followed by a final extension (72 °C, 10 min). The PCR protocol for Alphonse - Maisy was 94 °C for 2 min, 30 cycles of denaturation

(94 °C, 30 s), annealing (53 °C, 40 sec), and extension (72 °C, 40 s), followed by a final extension (72 °C, 10 min). PCR fragments were sequenced in both directions using an Applied Biosystems 96 capillary 3730xl DNA Analyzer at UC Berkeley sequencing facilities. Sequences were compared using Sequencher® software. Haplotypes were assigned to each maternal lineage.

Mitochondrial COI haplotypes of the two foundresses differed in 19 out of the 28 P-P+ figs. From each of these 19 figs, we sequenced (forward and backwards) the COI mitochondrial gene of 20 randomly chosen female, and 20 randomly chosen male offspring, to assign them to maternal lineage. Sequences were successfully obtained for a total of 339 female offspring from 19 figs, with 20 females in each of all but three figs. All sequenced female offspring could unambiguously be assigned to one of the two maternal lineages in each fig. Sequence quality from the male offspring was frequently poor, preventing us to use sequencing to assign males to maternal lineages.

To assign male offspring to maternal lineages and to distinguish between maternal lineages within each of the nine figs whose foundresses shared COI haplotypes (9 of 28 figs), we used three microsatellite loci that previously had been developed for this species: Pe77, Pe91, and Pe99 (Molbo *et al.* 2002). We determined fragment sizes using LIZ600® and an Applied Biosystems 96 capillary 3730xl DNA Analyzer at UC Berkeley sequencing facilities, and scored the peaks with Peakscanner® software. We obtained 16 scorable alleles at the Pe77 locus, 11 at the Pe91 locus and 19 at the Pe99 locus. The family produced by a singly mated haplodiploid foundress can receive up to three different alleles at each locus (two from her and one from her

haploid mate). We identified the alleles belonging to each maternal lineage by genotyping either five of each foundress's female offspring (if they could be identified by their COI haplotypes) or six of her sisters (if her COI haplotype was identical to the other foundress in her fig). Within each fig, private wasp alleles were available for at least one of the microsatellite loci, often two, which made manually sorting the offspring into maternal lineages straightforward. Only two male individuals (from two different figs) could not be assigned to a specific maternal lineage. In those two figs we based our analyses on the remaining 19 males.

Calculations and statistical methods

For each P-P+ fig we estimated (based on the 40 genotyped offspring) what proportion of the female or male offspring had originated from the P- and the P+ foundress, respectively. We then used this information to estimate the total number of offspring that each foundress produced in the P-P+ figs. For those figs in which all offspring had been counted and sexed we estimated the number of female offspring produced by the P- foundress for each fig: $P\text{-}_{P\text{-}P+F} = r_F \times F$, the number of male offspring produced by the P- foundress for each fig: $P\text{-}_{P\text{-}P+M} = r_M \times M$, and the overall number of offspring produced by the P- foundress for each fig: $P\text{-}_{P\text{-}P+} = r_F \times F + r_M \times M$ (equation 1). In these equations, F = the total number of female offspring in the fig, M = the total number of male offspring in the fig, r_F = the proportion of female offspring belonging to the P- foundress in each P-P+ fig, and r_M = the proportion of male offspring belonging to the P- foundress in each P-P+ fig. The latter two variables were estimated by genotyping a subset of 20 female and 20 male offspring from each fig as described above. We here assumed that the genotyped subset accurately represented the total offspring population of each sex.

We then compared the number of offspring that a P- foundress produced in a P-P+ fig ($P-_{P\text{-}P+}$) with what a P- foundress produced in a P-P- fig ($P-_{P\text{-}P-} = 0.5\times F + 0.5\times M$), and similarly compared the number of offspring that a P+ foundress produced in a P-P+ fig ($P+_{P\text{-}P+}$) with what a P+ foundress produced in a P+P+ fig ($P+_{P+P+} = 0.5\times F + 0.5\times M$). We here assumed that each of the two foundresses in P-P- and P+P+ figs contributed half of the total offspring in each fig. Similarly, the production of female and male offspring for a P- foundress in a P-P- fig could be calculated as: $P-_{P\text{-}P\text{-}F} = 0.5\times F$ and $P-_{P\text{-}P\text{-}M} = 0.5\times M$, and for a P+ foundress in a P+P+ fig: $P+_{P+P+F} = 0.5\times F$ and $P+_{P+P+M} = 0.5\times M$.

To include all P-P+ figs in the analyses, including those three where we did not have exact counts of male and female offspring but only counts of empty galls, we averaged the sex ratios observed in the 59 figs from which all offspring had been sexed, 0.87 (s.e.m.0.05) female and 0.13 (s.e.m. 0.05) male, and used those average sex ratios to produce estimates of how many offspring were produced by the P- and P+ foundresses of figs also with incomplete data: $P-_{P\text{-}P+} = 0.87\times T\times r_F + 0.13\times T\times r_M$ (Equation 2), and $P+_{P\text{-}P+} = T - P-_{P\text{-}P+}$ (Equation 3). Here, T is the total number of offspring (quantified as the number of empty galls) in each P-P+ fig. If these approximate equations are applied to figs for which the exact numbers of male and female offspring are known, they produce very similar estimates of total number of offspring per fig as the more exact equations, differing on average 0.1% and at most 3%.

To test whether the proportion of males and females in P-P+ figs that originated from the P- foundress was different from that originating from the P+ foundress, we used R version 2.11 to perform randomization tests. For each tree, the proportion of males or females originating from

the P- foundress was randomly switched with that originating from the P+ foundress in the same fig, and iterated 10 000 times to create a distribution as would be seen if there had been no difference between P- and P+ foundresses in their contribution to the brood. For each tree, the mean proportion from the original data was then compared with the created distribution to obtain a p-value. All other statistical analyses were done using SPSS 17.0. Whenever the assumptions of normality or equality of variances were not met, we used non-parametric statistical methods. All tests are two-tailed unless otherwise stated.

3. Results

The effect of treatment on seed numbers

P+ wasps pollinate both the flowers in which they lay their eggs, and flowers without eggs that will become seeds. We therefore expected P-P- figs to have no seeds, P-P+ to have an intermediate number of seeds, and P+P+ figs to have the most seeds. Experimental P-P- figs on both trees did not contain any seeds, except two figs that contained 3 and 1 seeds respectively (tree BS#1: n = 21, mean 0.14 seeds, s.e.m. 0.14; tree BCI#1: n = 3, mean 0.33, s.e.m. 0.33; Fig. 2.3). On tree BS#1, P+P+ figs contained marginally more seeds than P-P+ figs (P-P+: n= 23; P+P+: n = 17; one-sided t-test, $t_{38} = -1.52$, $p = 0.068$; Fig. 2.3). On tree BCI#1, P+P+ figs contained significantly more seeds than P-P+ figs (P-P+: n = 5; P+P+: n = 4; one-sided t-test, $t_7 = -2.39$, $p = 0.024$; Fig. 2.3). Note that the sample size on tree BCI#1 is very small.

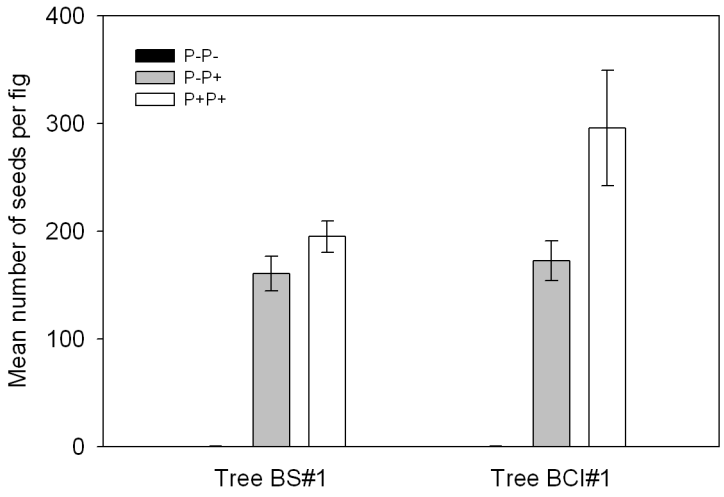

Figure 2.3. Mean number of seeds in P-P-, P-P+ and P+P+ figs from trees BS#1 and BCI#1. Error bars represent 1 s.e.m.

Partition of offspring within P-P+ figs

We tested whether the offspring of P-P+ figs were skewed towards the P+ foundress (predicted by individualized sanctions) or if each foundress contributed equally (predicted by modular sanctions). Within P-P+ figs, half of the offspring, or more, originated from the P- foundress (Fig. 2.4). P- and P+ foundresses did not differ significantly in the proportion of males or females they produced, on either of the trees (randomization tests: Tree BS#1 females: $p = 0.98$, males: $p = 0.99$; Tree BCI#1 females: $p = 0.12$; males: $p = 0.50$). For comparison, one-sample t-tests comparing the proportion P- males or females against 0.5 give almost identical results (except BCI#1 females are just significant at $p = 0.048$), but are not ideal to use since the data are proportions.

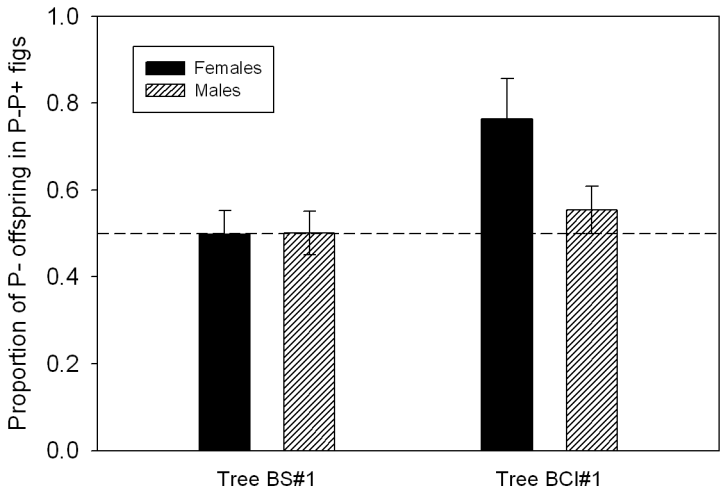

Figure 2.4. The mean proportion of female and male offspring that belonged to the P- foundress in P-P+ figs did not significantly differ from 0.5 (dotted reference line) on the two experimental trees. Error bars represent 1 s.e.m.

Comparison of offspring production per foundress depending on which kinds of foundresses share the fig

Next, we used equations 1, 2 and 3, as appropriate, to compare the number of offspring that a P- foundress produced in a P-P+ fig (P_{-P-P+}) with the number that a P- foundress produced in a P-P- fig (P_{-P-P-}); similarly, we compared the number of offspring that a P+ foundress produced in a P-P+ fig (P_{+P-P+}) with the number that a P+ foundress produced in a P+P+ fig (P_{+P+P+}). On both experimental trees, P- foundresses produced clearly and significantly more offspring when sharing a fig with a P+ foundress than when sharing a fig with another P- foundress (Tree BS#1: t-test, $t_{42} = -5.49$, $p = 2.1\times10^{-6}$; Tree BCI#1: t-test, $t_6 = -3.23$, $p = 0.018$; Fig. 2.5). Further, on tree BCI#1, P+ foundresses produced significantly more offspring when sharing a fig with another P+ foundress, than when sharing a fig with a P- foundress (t-test, $t_7 = -2.94$, $p = 0.022$;

Fig. 2.5b). However, on tree BS#1 P+ foundresses did not produce significantly more offspring in P+P+ figs than in P-P+ figs (t-test, $t_{38} = -0.59$, $p = 0.56$; Fig. 2.5a).

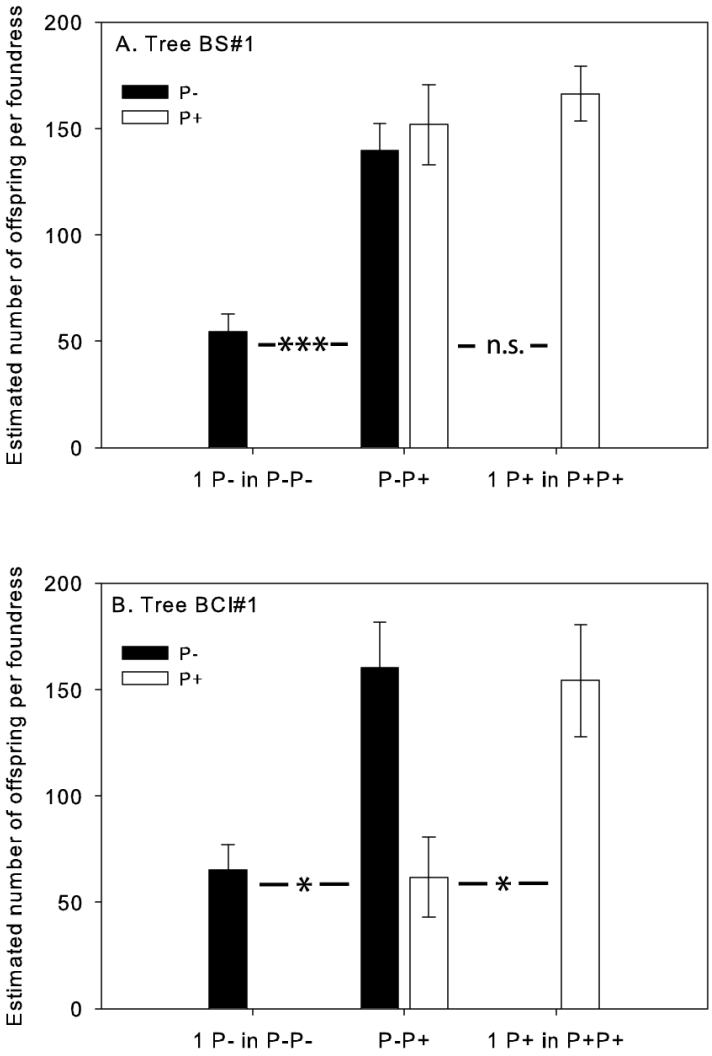

Figure 2.5. The mean estimated number of offspring that a foundress produced in the different types of figs on trees A) BS#1 and B) BCI#1. Error bars represent 1 s.e.m. Groups whose means are statistically different are labelled *: $p < 0.05$; ***: $p < 0.001$.

Differences in males and females

On tree BS#1, we had the exact numbers of male and female offspring from a subset of the figs (sample sizes P-P-: 17, P-P+: 21, P+P+: 13), which allowed us to examine how the number of male and female offspring of a specific foundress differed depending on the type of foundress with which she shared a fig. Similar to the overall offspring data, P- foundresses produced significantly more both females and males when sharing the fig with a P+ foundress (Females: Mann Whitney U-test, $Z = -3.27$, $p = 0.001$; Males: Mann Whitney U test, $Z = -2.75$, $p = 0.006$; Fig. 2.6). Also similar to the total offspring data, the type of sharing foundress did not influence the number of females or males that a P+ foundress produced on tree BS#1 (Females: Mann-Whitney U-test, $Z = -0.89$, $p = 0.38$; Males: Mann-Whitney U-test, $Z = 0.70$, $p = 0.70$; Fig. 2.6). On tree BCI#1, the number of figs in which we knew the exact number of male and female offspring was too low for meaningful statistical analyses (n: P-P- = 2, P-P+ = 2, P+P+ = 4), but the pattern was similar to that observed for total offspring numbers.

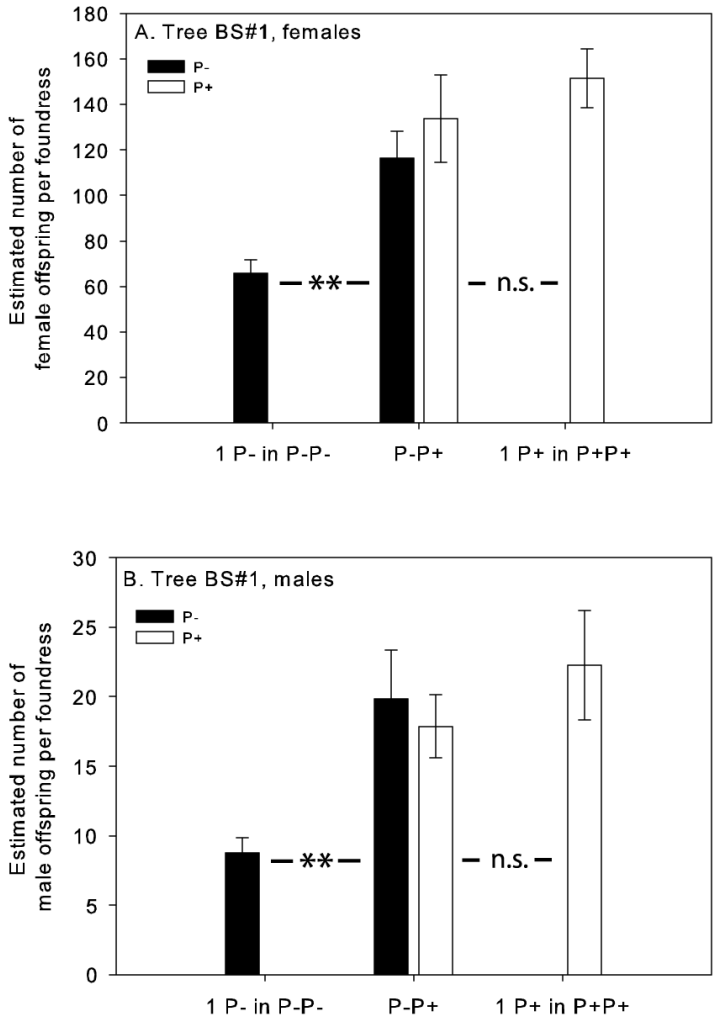

Figure 2.6. Mean estimated A) female and B) male offspring numbers belonging to the respective foundresses for the three treatment types in the subset of figs on tree BS#1 where exact counts of males and females were available. Error bars represent 1 s.e.m. Groups whose means are statistically different are labelled **: $p < 0.01$.

4. Discussion

The main conclusion that we can draw from our results is that offspring reducing sanctions in *Ficus nymphaefolia* act on the level on the entire fig, not on individual flowers inside the fig. Such modular sanctions above the level of individual wasps allow a cheating pollen-free wasp to free-ride on co-habiting pollinating foundresses. The pollen-free wasp can therefore enjoy the

benefits of pollination by the pollinating foundress(es) without herself paying any possible costs of pollination.

There was no support for the hypothesis that sanctions act on the flower level (hypotheses 2A & 2B, table 1), which predicts fewer offspring from P- wasps than from P+ wasps in P-P+ figs. Instead, within the P-P+ figs, P- wasps produced equal (tree BS#1) or a tendency to more (tree BCI#1) offspring than did P+ wasps. The most likely explanation for this result is that sanctions act on the fig level. There is a small possibility that flower level sanctions exist but that they were obscured by pollen spill-over (wasps occasionally pollinate flowers in which they have not laid eggs (Jandér unpublished), and in some fig species pollen tubes can grow across stigmas to neighbouring flowers (Jousselin and Kjellberg 2001)), but we doubt that pollen spill-over would be frequent enough to account for P- wasps having on average 50% or more of the offspring. Moreover, if pollen spill-over were that common, it would vitiate sanctions of individual flowers. Another possibility is that P- wasps might lay their eggs in flowers that are already pollinated (but not oviposited in) by P+ wasps. However, because this would restrict the number of flowers available for P- wasps, making it unlikely that P- wasps would have the 50% or more of the offspring in P-P+ figs that we found experimentally, we do not consider this a likely explanation.

Previous experiments with only a single P- or P+ wasp in each fig (Galil and Eisikowitch 1971; Nefdt 1989; Jousselin and Kjellberg 2001; Jousselin *et al.* 2003; Tarachai *et al.* 2008; Jandér and Herre 2010) have been unable to distinguish between the hypotheses that the reduced offspring numbers in unpollinated figs are due to i) unfertilized flowers being poorer food for growing wasp larvae (lack of endosperm) (Verkerke 1989), ii) unfertilized flowers are less likely to

successfully transform into wasp galls (Jousselin *et al.* 2003), or iii) figs without seeds receive less resources from the tree, and thereby also less resources for the growing wasp larvae. By showing that offspring reducing sanctions do not act at the flower level, our results contradict for this species both the hypothesis that fig wasp larvae would receive better nutrition from endosperm (fertilized flower structures) than from unfertilized flowers, and the hypothesis that fertilization increases the likelihood of successful gall formation. A remaining possibility is that not wasp number but wasp size could be affected by the lack of fertilization. Preliminary data do not show any difference in the size of offspring from P- or P+ foundresses in the P- P+ figs, further suggesting that fertilization of the flower in which the wasp develops does not affect wasp fitness, and a more thorough study is underway.

Sanctions act on the fig level then, and it is very clear from our data that foundresses in figs with some pollination (P-P+ and P+P+) produce many more offspring than foundresses in completely unpollinated figs. The most likely reason for this is that resources were allocated to the fig as a whole based on the overall pollination level, and the developing offspring in pollinated figs therefore had access to more overall resources than those in the P-P- fig.

Given that sanctions act on the fig level, is one pollinator in a fig sufficient to avoid sanctions (hypothesis 1A, table 1), or does resource allocation increase with increasing pollination levels (hypothesis 1B)? Our two different experimental trees suggest slightly different conclusions. On tree BS#1, wasps in P+P+ figs did not produce significantly more offspring than P+ wasps in P- P+ figs, which suggests that one pollinator inside a fig is sufficient to avoid sanctions (hypothesis 1A). However, on this experimental tree the average number of seeds in a P+P+ fig

was marginally but not significantly larger than that in P-P+ figs. Therefore, even if hypothesis 1B is true, on this tree, we could not necessarily expect P+ wasps in P+P+ figs to have significantly more offspring than P+ foundresses in P-P+ figs on this tree.

Tree BCI#1 showed a different pattern. On this tree, P+ wasps in P+P+ figs produced vastly more offspring than did P+ wasps in P-P+ figs. However, this pattern was driven by the unexpected result that within P-P+ figs, P- wasps produced the majority of the offspring (especially female offspring). The experiment on tree BCI#1 had a sample size of only five figs, and the result was partly driven by two P-P+ figs that had very high proportions of P- female offspring (95% and 100% respectively). This does not seem to be a side effect of the maternal lineage from which the P- foundresses originated: P- foundresses in these two extreme figs came from two different lineages, and these lineages also produced other, less skewed figs. Although naturally occurring P- foundresses could be expected to obtain some benefit (saving energy or pollination time) from not carrying pollen, most of those benefits would not be realized in artificially produced P- wasps such as were used in these experiments.

With the two trees showing somewhat different patterns, we cannot distinguish between hypotheses 1A and 1B. A cautious interpretation would be that the pollination level accomplished by one foundress might not be sufficient to completely avoid sanctions in this species, although only slightly more resources are added when adding the pollination efforts of another wasp. Fig species are likely to activate sanctions at different thresholds, perhaps dependent on factors such as fig size, the relative cost of mistakenly aborting a beneficial fig, and resource availability.

Although this study was limited to experimental figs with two foundresses, other foundress combinations, involving more foundresses per fig, are certainly possible. In *F. nymphaefolia*, 33 % of figs contain three or more foundresses. While it is likely that a pollen-free wasp in such a fig would suffer little or no sanctions, the number of offspring for each foundress will be reduced due to competition for oviposition sites (Herre 1989). For a pollen-free wasp, the likely ideal is to share a fig with one pollinating wasp, but not more.

We propose that the absence of flower-level sanctions, exhibited by *F. nymphaefolia*, can be extrapolated to *F. popenoei* and other closely related fig species, for two reasons. Firstly, *F. nymphaefolia* and *F. popenoei* are very closely related (Machado *et al.* 2005; Jackson *et al.* 2008). Secondly, all Panamanian fig species in the actively pollinated Urostigma group show the same types of sanctions, which differ only by degree. They all abort a proportion of unpollinated figs, and reduce wasp offspring numbers in unpollinated figs that mature (Jandér and Herre 2010). We see no reason to expect drastic differences in the precision of sanctions across species, as the basal mechanism of sanctions likely is the same across these species.

How likely might individual pollen-free wasps benefit from the free-riding possibility that fig level sanctions open up? It depends on both the frequency of pollen-free wasps and the frequency with which figs are colonized by more than one foundress. In *F. nympheafolia*, pollen-free wasps (NP-) are very rare; comprising only 0.3% of the population. So, any other wasp inside the fig is likely to be a pollen-carrier. Although 76% of wasps associated with *F. nymphaefolia* end up in figs with more than one foundress, where free-riding would be possible, 24% of wasps end up in single-foundress figs where wasps that do not pollinate suffer strong

sanctions (Jandér and Herre 2010). In contrast, in *F. popenoei* only 7% of wasps end up in single foundress figs, and sanctions against non-pollinators are milder (Jandér and Herre 2010). The remaining 93% of wasps end up in multiple foundress figs. Although pollen-free wasps are considerably more common in *F. popenoei* (5% of the wasp population), there might still be plenty of opportunities for free-riding. Thus, if sanctions are modular also in *F. popenoei*, pollen-free wasps would be able to escape sanctions most of the time. If there are fitness costs to carrying pollen and pollinating actively, free-riders might spread in the population until the benefits of not pollinating are balanced by the costs of sanctions (the likelihood of sanctions increases as the proportion of pollen-free wasps in the population increases). For a detailed mathematical evaluation of the balance between costs and benefits of cheating in the different Panamanian species, please see Jandér (model, MS).

Although sanctions have been described in other mutualistic systems (Pellmyr and Huth 1994; Jousselin *et al.* 2003; Kiers *et al.* 2003; Simms *et al.* 2006; Tarachai *et al.* 2008; Bever *et al.* 2009; Goto *et al.* 2010; Jandér and Herre 2010; Sachs *et al.* 2010), few have attempted to study the precision level of sanctions. Of course, systems where the only sanction type known is fruit abortion, such as in yuccas and *Glochidion*, suggest modular sanctions (Pellmyr and Huth 1994; Goto *et al.* 2010). Interestingly, in these systems the plant individual would derive no immediate benefit from selecting to rear cooperative rather than uncooperative moths, since the pollination of the plant's flowers is already done, and the moth larvae reared in the plant's fruits do not necessarily collect pollen from their host plant as adults. In contrast, fig wasps do collect pollen from their natal host plant, so rearing cooperative wasps has more direct benefits for fig trees,

implying that individualized sanctions would be more likely in figs than in yuccas and *Glochidion*.

In mutualisms between plants and nutrient-supplying root symbionts such as mycorrhizae and nitrogen-fixing rhizobia, plants would theoretically benefit from being able to impose individualized sanctions (Denison 2000; Bever *et al.* 2009; Friesen and Mathias 2010). In legumes, modular nodule level sanctions are already known (Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010), but a single nodule can be infected by multiple rhizobial strains, rendering nodule level sanctions less effective (Denison 2000; Friesen and Mathias 2010). In a recent empirical study (Sachs *et al.* 2010) the authors suggested that sanctions in the legume *Lotus* may be acting on the bacteroid level (individualized sanctions), although the definite calculations to distinguish between modular or individualized sanctions seem to not have been done on that dataset. Plants interacting with mycorrhizal fungi would theoretically also benefit from applying individualized sanctions, although to do so plants might have to apply sanctions on the level of individual cells. In an experimental study, *Allium* plants were able to selectively allocate resources to the more beneficial mycorrhizal fungi when fungal populations were clearly separated within the root system, but not when fungal populations were well mixed (Bever *et al.* 2009). Plants in that study therefore could not control resource allocation on the scale of individual arbuscules, but it is not clear whether plants allocated resources on the scale of whole roots or rootlets, and whether the scale of resource allocation in this species is finer or coarser than the distribution of different mycorrhizal populations in nature (Bever *et al.* 2009).

An interesting situation is found in the more complex behavioural mutualism between reef fish clients and their cleaning fish. Cleaners often work in pairs, and although it is theoretically possible that a cheated client could punish (chase) cheating cleaners individually, in practice a cheated client simply leaves the cleaning station (equivalent of modular sanctions) (Bshary *et al.* 2008). Interestingly, pairs of cleaners cheat less often than cleaners that work alone, seemingly because the male in the pair often punishes the female if she cheats (Bshary *et al.* 2008). In this system therefore the fellow symbiont, rather than the host, imposes the equivalent of individualized sanctions.

Different mutualistic systems therefore show different levels of precision in host sanctions. In each system, the precision of sanctions is likely to be determined by a balance between costs and benefits. For mutualisms where mixed interactions occur, the host will theoretically always benefit from more precise sanctions (Friesen and Mathias 2010). However, if there are costs to increasing sanction precision, important factors affecting its evolution will likely include the frequency of mixed interactions, the frequency and magnitude of cheating, and the relative cost of less precise (modular) sanctioning alternatives.

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CHAPTER 3

THEORETICAL EXAMINATION OF MECHANISMS THAT COULD MAINTAIN THE BEHAVIOURAL POLYMORPHISM IN POLLINATOR FIG WASPS

Abstract

Polymorphisms in natural populations are striking and intriguing. What mechanisms maintain these multiple morphs – are they adaptive, or maladaptive but repeatedly introduced through mutations? In the mutualism between fig trees and their pollinating fig wasps, wasps pollinate fig flowers and rear their larvae inside figs. Some fig wasp individuals do not carry pollen, and therefore cannot pollinate. Fig trees, in turn, are able to impose sanctions that reduce the fitness of pollen-free wasps. Therefore, being a pollen-free wasp can incur costs, but are there also benefits? Here I use mathematical models parameterized with empirical data to help distinguish between some of the hypotheses regarding this behavioural polymorphism in fig wasps. The large differences across fig species in the strength and likelihood of sanctions lead to different mechanisms being likely for maintaining the wasp polymorphisms in these closely related wasp species. Model estimates combined with field collected data support a mutation-selection balance maintaining the polymorphism in wasps associated with *F. citrifolia, F. nymphaefolia*, and *F. obtusifolia*, but reject it for wasps associated with *F. popenoei*. Instead, models estimating the level of benefits needed to maintain the pollen-free morph in each species suggest that this is a plausible option for wasps associated with the weakly sanctioning *F. popenoei*, but not the other species.

1. Introduction

Polymorphisms occur in most natural populations, and include differences in easily observed traits such as colour and body shape, and differences in less easily observed traits such as behaviours or enzyme forms. Polymorphisms have intrigued both theoretical and empirical researchers for centuries (Darwin 1859; Mendel 1865). Why are there multiple morphs; what prevents the less common morph from disappearing from the population? As a null hypothesis, polymorphisms could be maintained by mutation-selection balance, where mutations increase the frequency of the rare morph and selection decreases it (Falconer and Mackay 1996). Although mutation rates are typically too low to produce noticeable morph frequencies, an exception being rare human conditions such as haemophilia (Haldane 1935; Vogel and Motulsky 1997), in highly inbred populations it is not unrealistic that mutations could be responsible for rare morphs (Falconer and Mackay 1996).

Alternatively, various types of fitness benefits could maintain a polymorphism. A rare morph without fitness benefits per se could be maintained by a heterozygote advantage, for example individuals heterozygous for sickle-cell anemia are more resistant to malaria, whereas homozygotes get severely ill (Allison 1954). It is also possible that all morphs have fitness advantages, but under different circumstances: 1. Under negative frequency dependent selection, a morph's fitness is negatively correlated with its frequency in the population (Ayala and Campbell 1974). For example, fruit fly larvae have two types of foraging behaviour – either sedentary or roving – and each morph does best when it is rare in the population (Fitzpatrick *et al.* 2007). At the equilibrium frequency of morphs, both have equal fitness (Maynard Smith

1982). 2. The different morphs could also result from a strategy chosen under certain environmental/external conditions (often called polyphenism), such as tadpoles developing into different morphs depending on whether predators are present or not (McCollum and Van Buskirk 1996), or under certain bodily/internal conditions (often called conditional strategy), such as dung beetle males developing into big-horned or small-horned morphs depending on their larval size (Emlen 1994). In this case, morphs do not necessarily have equal fitness on average, but their fitness at the switchpoint of the environmental gradient is equal (Gross 1996).

Distinguishing between the different alternative mechanisms is not straightforward in many polymorphic systems. In this study I use some simple mathematical models combined with empirical data from natural populations to estimate which mechanism(s) most likely maintain the behavioural polymorphism of pollen-carrying and pollen-free wasp individuals in the fig tree – fig wasp mutualism.

Relevant natural history

Fig trees and their pollinating wasps are completely dependent on each other for reproductive success – fig wasps lay their eggs in fig flowers while pollinating. This mutualism is ancient and diverse with over 700 known fig species (Rønsted *et al.* 2005). Each species of fig tree typically interacts with only one or two pollinating wasp species, sometimes morphologically indistinguishable (cryptic species; Molbo *et al.* 2003; Haine *et al.* 2006). The description below of wasp behaviour is relevant to actively pollinating wasp species. All empirical data in this paper come from natural fig and wasp populations near the Panama Canal, Panama, with species

characteristics in table 3.1. For simplicity I will use the names of the fig species also when referring to the associated wasp species (e.g. *F. popenoei A* refers to *P. gemellus A*).

Table 3.1. Summary of species characteristics for the four studied fig species and their respective pollinating wasp species. *F. = Ficus, P. = Pegoscapus*. Data from [1] (Berg 1989; Wiebes 1995; Molbo *et al.* 2003), [2] (Herre 1989), [3](Herre 1988), [4] current study.

Fig species	**Pollinator species**[1]	**Mean flower number (s.e.m.)**[2]	**Number of offspring for a single foundress (s.e.m.)**[3]	**Mean foundress number**[4]
F. citrifolia	*P. tonduzi*	325 (5)	163.3 (3.5)	1.40
F. nymphaefolia	*P. piceipes*	675 (14)	247.2 (5.4)	2.15
F. obtusifolia	*P. hoffmeyeri A (common)* *P. hoffmeyeri B (rare)*	976 (16)	335.0 (10.9)	1.32
F. popenoei	*P. gemellus A (rare)* *P. gemellus B (common)*	1124 (21)	174.0 (6.2)	3.85

Fig flowers are located on the inside of hollow ball-like inflorescences named syconia, hereafter called figs. There are typically several hundred tiny uniovulate flowers inside a fig; in monoecious fig species each flower can support the development of either a single wasp (approximately the size of a fruit fly) or a seed. Fig wasps thus develop inside galled fig flowers. When a fig is nearly mature, male wasps emerge first from their galls, and crawl around inside the dark fig searching for galls containing females. Each male will open a female gall with his powerful jaws, mate with the female while she is still inside her gall, then move on to the next female gall. Each female emerges from her gall, actively searches for and locates a now mature male flower with stamens (within the same fig), then uses her front legs to scoop up pollen grains and fills the two pollen pockets on either side of her thorax (Frank 1984; Jandér unpublished data). Each female will then exit the fig through an exit tunnel (dug by the males), and fly upwards towards the light to gain height (Ware and Compton 1994; Ware and Compton 1994). A

fig wasp can fly with the winds above the canopy for long distances (average distance 10 km; Nason *et al.* 1998) until she detects the specific scent of a receptive tree of her own fig species (Hossaert-McKey *et al.* 1994). She will then drop down, backtrack to find the source tree, and locate a receptive fig to enter (Ware and Compton 1994; Ware and Compton 1994). Wasp lifespan outside their natal fig is typically less than 1-3 days (Kjellberg *et al.* 1988; Harrison and Rasplus 2006; Jandér unpublished data), and less than 1% of female wasps make it to a flowering fig (Herre 1989).

One or more female wasps (now called foundresses) enter each flowering fig through a narrow one-way opening, called the ostiole, then oviposit and pollinate following strictly stereotypic behaviour: wasps first search for a suitable flower, then oviposit and pollinate, and then start the search again for a new flower within the fig (Frank 1984; Jandér unpublished data). A wasp lays up to several hundred eggs in the fig before she dies, and is often limited not by egg numbers but by lifespan (Jandér unpublished data) or flower availability. Female fig wasps carry all eggs from birth (Copland and King 1973). In some fig species foundresses are known to exit the first fig after a while and enter a new fig (Moore *et al.* 2003) – in the species studied here such behaviour is very rare and instead foundresses die inside the first fig they enter. Therefore, the number of dead foundress bodies is a good estimate of the number of foundresses that contributed to the brood (for an exception, see Molbo *et al.* 2004). In single foundress figs, wasp lifetime reproductive success can be easily quantified by counting the number of wasp offspring emerging from a fig (Herre 1989).

While most wasp individuals of each actively pollinating wasp species carry pollen, some do not - their pollen-pockets are empty. This pollen-free (P-) morph is rare (0.3-5% depending on species), but occurs in all six actively pollinating fig wasp species studied so far in Panama (Jandér and Herre 2010). The four corresponding fig tree species show plant sanctions that lower the fitness of wasps that do not pollinate – trees selectively abort unpollinated figs, and fewer wasp offspring develop in unpollinated than in pollinated figs. Sanction strength varies across fig species (Jandér and Herre 2010).

What mechanisms balance the fitness cost that sanctions impose, and maintain the pollen-free trait in the wasp populations? For this system, there are several alternative mechanisms (table 3.2). Some mechanisms could be ruled out if we knew whether the pollen-free trait is heritable (passed from mother to offspring) or not, and this is currently under study. If the P- trait is heritable, possible mechanisms include mutation-selection balance and frequency dependent selection on a genetic polymorphism (Maynard Smith 1982) (Table 3.2). If the P- trait is not heritable, possible mechanisms include frequency dependent selection on a mixed strategy (Maynard Smith 1982), that it is a chosen strategy under certain environmental conditions, or simply that P- wasps are environmentally caused "mistakes" (for example if the pollen supply in a fig ran out before all wasps had collected their share). Likewise, some mechanisms could be ruled out if we knew whether there were fitness benefits associated with the pollen-free trait, such as saving energy due to not carrying pollen or not doing pollination movements (Table 3.2). However, due to fig wasp life history and the rareness of the pollen-free morph, this is difficult to study. Instead, here I will use a few simple mathematical models with parameters set using species-specific empirical data for four different fig species and their respective wasps, to

investigate whether some mechanisms can be ruled out by for example requiring unrealistically large mutation rates or unrealistically large relative fitness benefits in order to explain the prevalence of pollen-free wasps found in nature.

Table 3.2. In a polymorphic wasp population with phenotypes P+ (pollen-carrying; common) and P- (pollen-free; rare), with negatively frequency dependent payoffs for phenotype P-, what mechanisms could explain the prevalence of P-?

	Is phenotype P- heritable?	
Does phenotype P- incur benefits?	Yes (genetic polymorphism)	No (genetic monomorphism)
Yes	I: Frequency dependent selection and genetic polymorphism (alternative strategies) with equal fitness at ESS proportions[a].	IIA: Frequency dependent selection and mixed strategy at ESS proportions[a], OR IIB: Chosen strategy under certain environmental or bodily conditions[c].
No	III: Mutation-selection balance[b]	IV: Environmentally caused mistakes[c]

[a] Needed benefit levels quantified in section 2c.
[b] Needed mutation rates quantified in section 2b.
[c] Addressed in an ongoing study for *F. popenoei*.

2. Results -- The models

2a. Fitness calculations only accounting for costs

In these fig species, single foundress fig wasps that do not pollinate suffer fitness costs due to sanctions (Jandér and Herre 2010). While sanctions by far are strongest when there is no pollinating foundress in the fig (only P-), a recent experimental study suggests that intermediate levels of pollination (1 P- and 1 P+ foundress sharing a fig) also might be associated with sanctions, although much milder than when there are no pollinators at all (Jandér *et al.* MS). The average expected fitness cost due to sanctions for a P- wasp will therefore depend both on the

strength of sanctions in the particular fig species of interest, and on the likelihood of entering a fig where no or only one other wasp pollinates (a function of the likelihood of entering a fig with *n* foundresses, and the prevalence of P- wasps in the population, both species-specific). To calculate the fitness costs for an average P- wasp within a species, I first make some assumptions: 1. If there are multiple foundresses in a fig, offspring are divided equally among them. 2. Foundresses in a fig are unrelated. This is reasonable given the distances that wasps disperse (Nason *et al.* 1998). 3. Wasps within the same pollinator type (eg. P-) are assumed to have equal oviposition rates, but the P- rate may differ from the P+ rate. 4. When pollinators of more than one species interact with a single fig species, frequencies of P- wasps are identical in the two cryptic wasp species (although we do not know exact frequencies, we do know that P- wasps are present in both cryptic species (Jandér and Herre 2010)). 5. In my calculations I first make the assumption that one P+ inside a fig is sufficient to avoid sanctions (I call this 1P+S from "1 P+ sufficient"). Then, I change the models slightly to allow sanctions to also act (mildly) on figs with intermediate pollination levels (1 P+ in a fig), but figs with two or more P+ do not suffer sanctions (I call this 2P+S). I will present results from these models side-by-side for comparison.

In the below model, fitness (number of offspring) for a focal P- wasp, W_{P-}, is therefore calculated as the (probability of entering a fig with (n-1-x) P- wasps and x P+ wasps) × (the expected offspring from this combination). The probability of ending up in a fig with n foundresses is f_n. Of these n wasps, (n-1-x) will be P- wasps (proportion q in the population) and x will be P+ wasps (proportion (1-q) in the population). The proportion of figs for each foundress

constellation is given by the formula $f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x$ when the focal wasp is a P-, and $f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)}$ when the focal wasp is a P+.

The probability f_n is different for each species, and given by field data – I had previously collected figs within a week after pollinator arrival, and counted the number of dead foundress wasps in each fig (Jandér and Herre 2010). These data allow me to calculate both the proportion of figs that have *n* foundresses (foundress distributions), and the proportion of foundresses that end up in figs with *n* foundresses, f_n, given that the wasp reaches a receptive fig (Jandér and Herre 2010).

In the 1P+S model (equations 1a & 2a), P- wasps do not pollinate figs and unpollinated figs suffer lower offspring numbers due to an increased likelihood of abortions and lower offspring numbers (Jandér and Herre 2010). Therefore the expected number of offspring in figs with only P- wasps ($x = 0$) is multiplied by the sanctions factor r_x where $r_0 < 1$. To calculate r_x I used data from our previous field experiments, which estimated the fitness of a single-foundress pollen-free wasp relative to that of a pollen-carrying wasp for these four fig species (Jandér and Herre 2010). In this basic all-or-nothing 1P+S model, there is a fitness cost only if there is no pollination ($r_0 < 1$); having one or more pollinators in the fig is sufficient to avoid sanctions ($r_{\geq 1} = 1$). This model reflects the findings from on one of two *F. nymphaefolia* trees in which replicate experimental figs were manipulated to include two foundresses in each of the following arrangements: P-P-, P-P+, or P+P+ (Jandér *et al.* MS).

In the more gradual 2P+S model (equations 1b & 2b), offspring reducing sanctions are strong in figs with no pollinator, mild in figs with 1 P+ and absent in figs with 2 P+. This model reflects the experimental findings from the second of the two studied trees in *F. nymphaefolia* (Jandér *et al.* MS), and is consistent with the hypothesis that resource allocation increases with pollination level. Abortions on the other hand (an all-or-nothing response) occur only when a fig has no P+ wasps at all ($x = 0$). For this model, I split up the sanctions factor r_x into its two components so that $r_x : r_{ax} \times r_{ox}$, where r_{ax} is due to abortions, and r_{ox} due to offspring reduction. The abortion factor r_{ax} is less than one when $x = 0$, but one otherwise (exact values from Jandér and Herre 2010). The offspring reducing factor r_{ox} is here calculated somewhat differently from the way it is calculated in model 1P+S. To estimate the values of r_{ox} for $x = 0$ and $x = 1$, I calculated what proportion of laid wasp eggs (estimated as wasp offspring numbers + empty galls from which wasps failed to develop (known as bladders; Tarachai *et al.* 2008)) developed into adult wasps in single foundress P- and P+ figs respectively (Jandér unpublished). Values of r_{ox} when $x = 0$ are very similar to the relative offspring numbers in Jandér & Herre 2010. Values of r_{ox} when $x = 1$ are around 0.8-0.9 depending on species (Jandér unpublished), and are consistent with what was found in the experimental two-foundress P-P+ or P+P+ figs in *F. nymphaefolia* (Jandér *et al.* MS). In this model, I assumed that figs with 2 or more P+ do not suffer any sanctions (also consistent with results in Jandér *et al.* MS).

In both these models all foundresses in a fig share the total potential offspring (t_n) equally. For these species, t_n values have previously been quantified in field collected figs (Herre 1988; Herre 1989). Typically, t_n increases with increasing number of foundresses inside a fig, and plateaus at

a species specific maximum (Herre 1989). The variables and parameters used in any of the models are specified in Table 3.3.

For the all-or-nothing 1P+S model:

$$W_{P-}(q) = \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot \frac{t_n}{n} \cdot r_x \qquad (1a)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot \frac{t_n}{n} \cdot r_x \qquad (2a)$$

And for the gradual 2P+S model:

$$W_{P-}(q) = \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot \frac{t_n}{n} \cdot r_{ax} \cdot r_{ox} \qquad (1b)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot \frac{t_n}{n} \cdot r_{ax} \cdot r_{ox} \qquad (2b)$$

Table 3.3. Description of variables and field-collected species-specific parameters used in any of the models in this paper. Sources are: [a] (Jandér and Herre 2010), [b] Jandér unpublished, [c] Jandér *et al.* MS, [d] (Herre 1988), [e] (Herre 1989).

Variable	*Description*
n	The number of foundresses in a fig.
q	The proportion of naturally occurring P- wasps in the population.
W_{P-}	The fitness (expected number of offspring) for a P- wasp.
W_{P+}	The fitness (expected number of offspring) for a P+ wasp.
x	The number of P+ wasps in a fig.
Species specific parameter	
f_n	The probability for a wasp to end up in a fig with n foundresses given that she enters a fig[a,b].
k	The relative likelihood of arrival at a flowering tree for a P- wasp compared to a P+ wasp.
m	The maximum recorded number of foundresses for a certain fig species[b].
q_{field}	The proportion of wasps of the pollinating species that are P- in natural populations[a].
r_x	The expected proportion of offspring developing from a fig with x pollinating foundresses, taking both fig abortions and offspring number reductions into account[a].
r_{ax}	The expected proportion of figs that mature, depending on x[a].
r_{ox}	The expected proportion of offspring that mature to adulthood, depending on x[b].
s	The selective disadvantage for a P- wasp compared to a P+ wasp.
t_n	The total number of offspring emerging from a fig with n foundresses[d,e].
t_{max}	The maximum total number of offspring emerging from a fig[e].
u_{ihf}	The mutation rate (taking inbreeding, haplodiploidy and selection on females into account) per locus per generation required to explain q_{field}.

Results

Equations 1a, 1b, 2a and 2b describe how fitnesses of the two morphs vary with an increasing proportion of pollen-free wasps (q) in the wasp populations when only costs are taken into account (Fig. 3.1). In the 1P+S model, W_{P+} is not affected by the frequency of P- wasps since one pollinator (minimally the focal P+ wasp) is enough to avoid sanctions. In the 2P+S model, W_{P+} decreases slightly when P- wasps become more common in the population, since P+ wasps

too suffer from the slight offspring reduction in figs with only 1 P+. In both models and for all species, W_{P-} decreases as the proportion of P- wasps increases in the population, due to a larger probability of sharing the fig with only other P- wasps and thereby suffering sanctions (and in model 2P+S, also a larger probability of sharing the fig with only 1 P+) (Fig. 3.1). Therefore, there is negative frequency dependent selection acting on P- wasps.

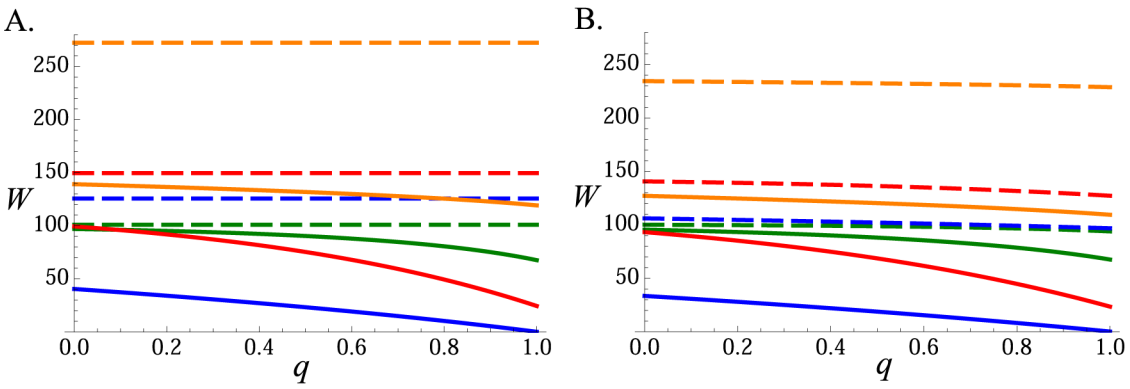

Figure 3.1. Graphs showing how expected female wasp fitness, *W,* varies with the proportion of P- wasps in the population, *q,* when only costs due to sanctions and no benefits are taken into account, for wasps associated with four different fig species. These graphs show absolute, not relative fitness, and expected offspring numbers therefore vary across species. A) Model 1P+S (equations 1a & 2a), B) Model 2P+S (equations 1b & 2b). Legend: W_{P+} dashed, W_{P-} solid. Orange: Wasps associated with *F. obtusifolia*, Red: *F. nymphaefolia*, Blue: *F. citrifolia*, Green: *F. popenoei*.

I can also use equations 1a and 2a or 1b and 2b to derive the coefficient of selection, s, for each species (based only on costs), which will be used in the next section. For each species I set W_{P+} = 1 and W_{P-} = 1-*s*. The selection coefficient *s* increases with *q* for all species, but more so for wasps on fig species with larger average foundress numbers (Fig. 3.2). When *q* = 0, only the

focal wasp is P-, all other wasps are P+. Here, P- wasps will be affected by sanctions only when they are single foundresses, and s (when $q = 0$) for each species is therefore a function of both sanction strength and of the likelihood of being a single foundress. As q increases, the likelihood of sharing a multi-foundress fig with only other P- wasps increases, which increases s. When $q = 1$ all wasps are P- wasps, and all these P- wasps experience sanctions because they cannot hide among P+ wasps. At that point, s is merely a function of sanction strength, and its values are therefore are identical to what was experimentally determined in single-foundress figs (Jandér and Herre 2010). These higher values of s are also what would be expected if sanctions were on the level of individual flowers within a fig (see Jandér et al. MS).

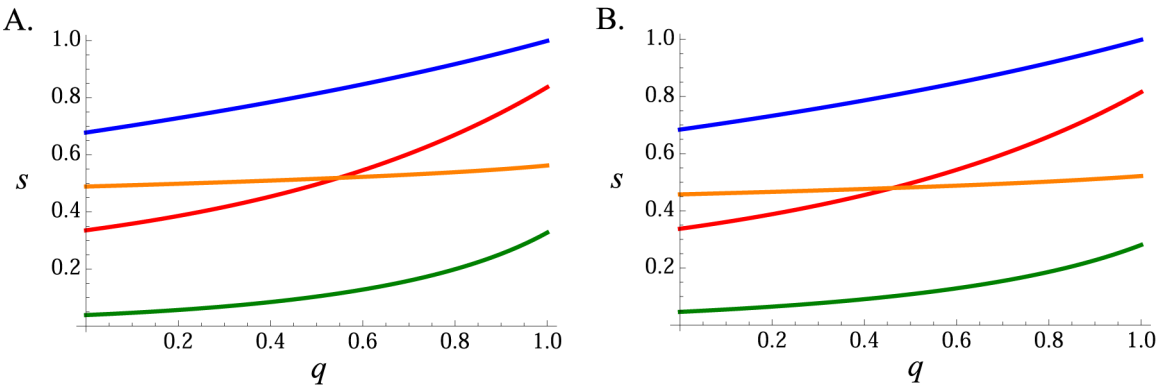

Figure 3.2. Selection coefficient s, based only on costs, as a function of the proportion of P- wasps in the population, q, for wasps associated with four different fig species (see text). A) Model 1P+S (equations 1a & 2a), B) Model 2P+S (equations 1b & 2b). Legend: Orange: Wasps associated with *F. obtusifolia*, Red: *F. nymphaefolia*, Blue: *F. citrifolia*, Green: *F. popenoei*.

In the next two sections, I will use the proportion of P- wasps that we have measured in the field, q_{field}, for each fig species (Jandér and Herre 2010). If I assume that the system is in equilibrium, then q_{field} represents the equilibrium q, and will allow me to calculate either what mutation rate is

needed (under a selection – mutation balance), or what level of fitness benefits are needed (under a cost-benefit balance), to maintain this equilibrium.

2b. Mutation – Selection balance model

In this model I balance the selection against P- wasps (due to sanctions, as modelled by either equations 1a and 2a, or 1b and 2b) with mutations. My goal is to see if the mutation rates that explain q_{field} are reasonable for the different species.

I here make the following additional assumptions:

1. The pollen-free trait is heritable (this is currently under study).

2. Migration is not important (either because the population is large and migration small, or because gene frequencies are similar in immigrants).

3. For simplicity, in this model I assume that the suite of required pollination behaviours are determined by one locus with a dominant allele A, and a recessive allele a; such that female P+ wasp have the alleles AA or Aa, and P- wasps have the fully recessive alleles aa. The allele a is present in the population in frequency p. Assuming that the pollen-free trait is determined by a single recessive allele is conservative. It is more likely that the pollen collecting behaviour is controlled by multiple genes, and losing any one of them would lead to loss of the pollen collecting behaviour. Also, in reality it is not required that mutations create the specific allele a – any mutation that destroys the function of the allele A would lead to loss of the pollen collecting behaviour (Van Dyken *et al.* 2011). In both cases, the required mutation rate would be lower in reality than what is calculated in this model.

Basic model

If mutations are fully balancing the selection against P- wasps, then selection against the a alleles will equate the mutation rate to the a alleles. For our fully recessive allele a, the equilibrium equation can be written as

$$u(1-p) - vp = \frac{sp^2(1-p)}{(1-sp^2)} \qquad (3)$$

where p is the equilibrium frequency of allele a in the population, u is the mutation rate per generation to allele a, v is the rate of mutation from allele a to allele A, and s is the selection coefficient (Falconer and Mackay 1996). When p is small, this expression can be simplified to $u = sp^2$ (Falconer and Mackay 1996; Van Dyken *et al.* 2011). This formula does however not account for the facts that fig wasps are both haplodiploid and inbred, and that selection would only act on females, and therefore needs to be adjusted.

Adjusting for inbreeding

Inbred populations at evolutionary equilibrium contain more homozygotes than do outbred populations. At a two-allele locus in an inbred population, with alleles present in frequencies p and $(1-p)$, homozygotes are present with frequencies $p^2 + p(1-p)F$, and $(1-p)^2 + p(1-p)F$, respectively, where F is the inbreeding coefficient (Falconer 1996, p 62). Since we assume that P- wasps are homozygotes for a, then their frequency in the population equals $p^2 + p(1-p)F$ (equation 4).

Adjusting for haplodiploidy and female-limited selection

For a recessive allele in a haplodiploid species (males haploid) where the selection acts only on females, mutational equilibrium occurs when

$$u = \frac{2p^2 s}{3} \qquad (5)$$

where p is the equilibrium frequency of allele a in the population, u is the mutation rate per generation to allele a, and s is the selective disadvantage incurred by homozygous females (Crozier 1976; Werren 1993).

The calculations

I then combine equations 4 and 5 to reach an estimate of the required mutation rates u_{ihf} (u taking into account inbreeding, haplodiploidy and selection only on females) that are required to reach the levels of pollen-free wasps that we observe in natural populations (q_{field}) of each species. In the calculations I use empirically derived values of F and s for each wasp species. The selection coefficient s is calculated using equations 1a and 2a (1P+S) or 1b and 2b (2P+S) respectively. The inbreeding coefficient F has been estimated directly using microsatellites for four of the fig wasp species (Molbo *et al.* 2004); for the remaining two species, F can be estimated indirectly using the formula $F = 1/(4n-3)$ (equation 6) where n is the harmonic mean of foundress numbers for each species (Hamilton 1979; Herre 1987) (table 3.3). The two available data sets of foundress number distributions in these fig species (Herre 1989; Jandér unpublished data) differ somewhat in that the Jandér dataset generally has slightly higher foundress numbers. Therefore I calculated two different foundress-based F values for each of these species, one from each data

set. In *F. nymphaefolia*, the two *F* estimates are very similar, whereas in *F. citrifolia* they differ a bit, but the different *F* estimates still produce rather similar estimates of u_{ihf} (Fig. 3.3, Table 3.4). Generally, for each species, u_{ihf} decreases as *F* increases (Fig. 3.3). Within each species, even moderate changes in *F* would not change u_{ihf} dramatically (Fig. 3.3).

Table 3.4. Species specific input and output values of the mutation-selection balance model. The values of *F* are determined from either the harmonic mean of number of foundresses in a fig (sources [a]Jandér unpublished and [b]Herre 1989), or from [c]microsatellite data (Molbo *et al.* 2004). For comparison I here show both $s(q_{field})$, which is *s* determined using the q_{field} values for each species, and $s(q=0)$, which is *s* determined for $q = 0$. The results in this table are calculated using $s(q_{field})$. The mutation rate u_{ihf} is that required for the model to reach q_{field} for each species, taking into account inbreeding, haplodiploidy and selection on females only. For *F. citrifolia* and *F. nymphaefolia,* where the two different data sets produced different *F* values, a u_{ihf} value is calculated for each *F* estimate. A separate value of u_{ihf} is calculated for each of the cryptic wasp species in *F. obtusifolia* and *F. popenoei.*

			Model 1P+S			Model 2P+S		
Fig species	**Wasp species**	***F***	$s(q_{field})$	$s(q=0)$	u_{ihf}	$s(q_{field})$	$s(q=0)$	u_{ihf}
F. citrifolia	*P. tonduzi*	0.571[a] – 0.708[b]	0.678	0.678	8.67×10^{-6} to 5.64×10^{-6}	0.685	0.684	8.73×10^{-6} to 5.69×10^{-6}
F. nymphaefolia	*P. piceipes*	0.300[a] – 0.317[b]	0.336	0.335	2.35×10^{-5} to 2.12×10^{-5}	0.338	0.337	2.36×10^{-5} to 2.13×10^{-5}
F obtusifolia	*P hoffmeyeri A* (common)	0.848[c]	0.489	0.489	4.35×10^{-5}	0.458	0.457	4.06×10^{-5}
	P hoffmeyeri B (rare)	0.877[c]	0.489	0.489	4.07×10^{-5}	0.458	0.457	3.80×10^{-5}
F popenoei	*P gemellus A* (rare)	0.404[c]	0.0425	0.0387	3.26×10^{-4}	0.0504	0.0463	3.56×10^{-4}
	P. gemellus B (common)	0.169[c]	0.0425	0.0387	7.66×10^{-4}	0.0504	0.0463	8.35×10^{-4}

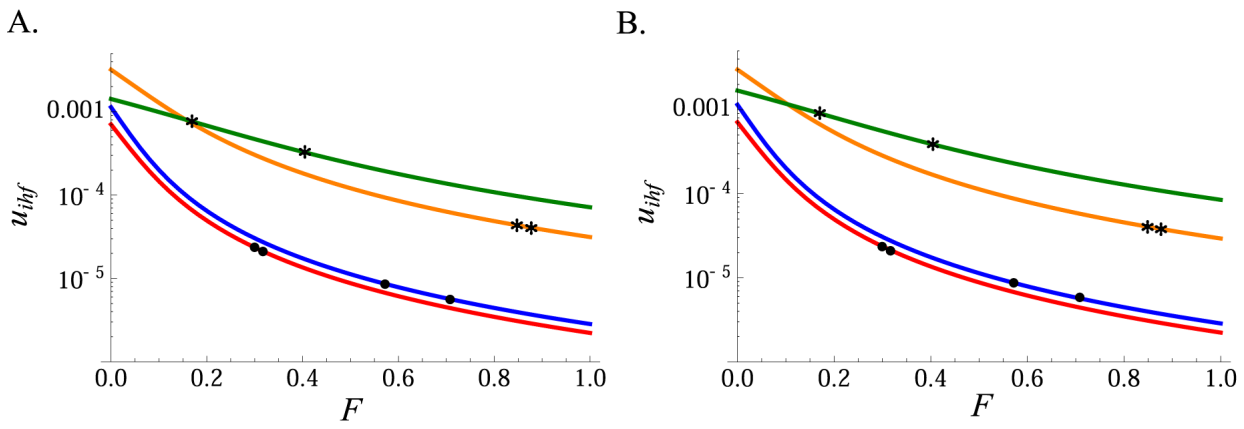

Figure 3.3. The mutation rate, u_{ihf}, required to explain the proportion of NP- wasps found in each population, depending on the inbreeding coefficient, *F*. The curve for each species is based on its empirically determined $s(q_{field})$ (selection coefficient) and q_{field} (proportion of NP- wasps in natural populations). A) Model 1P+S (*s* based on equations 1a & 2a), B) Model 2P+S (*s* based on equations 1b & 2b). Legend: Orange: Wasps associated with *F. obtusifolia*, Red: *F. nymphaefolia*, Blue: *F. citrifolia*, Green: *F. popenoei*. The black symbols mark empirically determined values for *F* based on microsatellite data (asterisks: *F. obtusifolia, F. popenoei;* two cryptic wasp species each), or on foundress number distributions in natural populations (black dots: *F. citrifolia, F. nymphaefolia*; one wasp species each with values from two different data sets, see text).

Results

The resulting u_{ihf} values are low and, for most of the species, within a range, 10^{-5} to 10^{-6}, that is thought of as common mutation rates per locus per generation (Falconer and Mackay 1996; Nachmann and Crowell 2000; Haag-Liautard *et al.* 2007; Lynch 2010) (table 3.4). However, the u_{ihf} values required to explain P- frequencies in the two wasp species associated with *F. popenoei* are an order of magnitude higher than those required for the other species.

Testing if mutations can explain the observed prevalence of P- wasps

Is it reasonable to assume that the q_{field} that we observe are due to mutation alone in these six wasp species? To test this hypothesis I calculated mutation- based estimates of q, q_{mut}, using

equations 4 and 5 and the empirically derived values of $s(q=0)$ and F (the mean value of F when two values were available for a wasp species) for the set mutation rates of 10^{-6}, 10^{-5}, and 10^{-4} (a reasonable upper range of plausible mutation rates, erring on too high). I then compared these q_{mut} with my field-collected q_{field} for each wasp species to see if there was any overlap (Fig. 3.4). The 95% confidence intervals about the population means of the field collected data were calculated using bootstrap (resampling with replacement) in Poptools with 10 000 iterations, and then bias corrected using the method described in (Quinn and Keough 2002 equation 2.13).

I report the results when using values of s from both model 1P+S, and model 2P+S. The selection coefficient s does increase somewhat with q, but in these calculations I chose to use the values of s when $q = 0$, which is not much different from s at the field-determined levels of q_{field} (Fig. 3.2, Table 3.4). However, this method will allow my mutation-based estimates of q, q_{mut}, to be independent of the field-determined q, q_{field}, for each species, thus removing any possibility of mathematical circularity. Using $s(q=0)$ instead of $s(q_{field})$ changes the results only slightly, and in the direction of making it harder to reject the mutation hypothesis. For example, in the calculation for model 1P+S where the difference between $s(q=0)$ and $s(q_{field})$ is the largest, *F. popenoei* for a u set to 10^{-4}, q_{mut} calculated using $s(q_{field})$ is 2.61% whereas q_{mut} calculated from $s(q=0)$ is only slightly higher at 2.74%, but still well below the observed q_{field} of 5%. The corresponding values for model 2P+S are 2.38% for $s(q_{field})$, and 2.49% for $s(q=0)$.

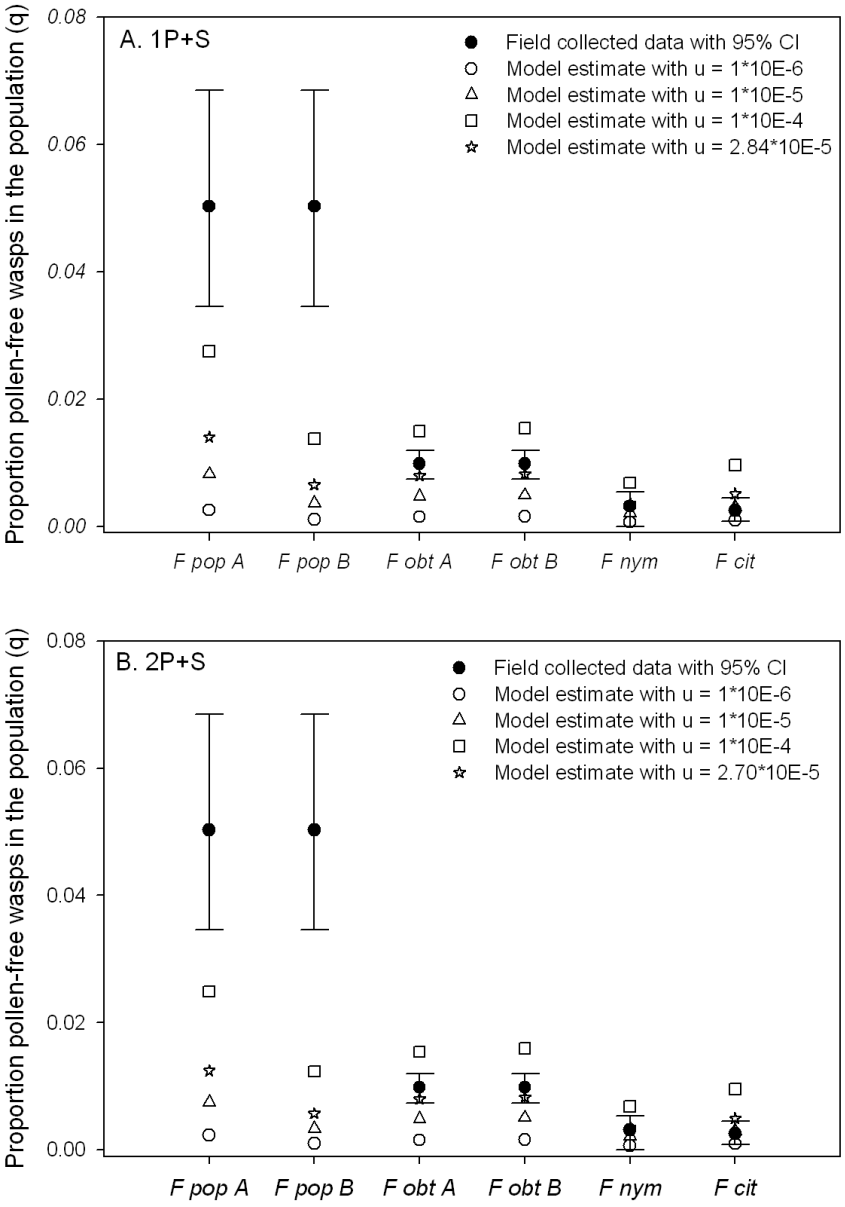

Figure 3.4. Comparison of *q*, the proportion pollen-free wasps in natural populations, as observed in the field (with 95% CI) and as estimated by a mutation-selection balance model with varying mutation rates. A) Model 1P+S (calculated with *s* based on equations 1a & 2a), B) Model 2P+S (*s* based on equations 1b & 2b).

In both models 1P+S and 2P+S, mutation rates of 10^{-6} and 10^{-5} give estimates of q_{mut} that are low but within the 95% CI for wasps associated with *F. citrifolia* and *F. nymphaefolia*, but too low

for *F. obtusifolia* and much too low for *F. popenoei* (Fig. 3.4). In both models, q_{mut} estimates based on $u = 10^{-4}$ are too high for all wasp species except those associated with *F. popenoei*, for which they are still much too low. It therefore seems that mutations are a plausible cause of q_{field} for wasps associated with *F. citrifolia*, *F. nymphaefolia,* and *F. obtusifolia*, but in *F. popenoei*, mutations alone cannot explain the relatively high prevalence of P- wasps observed in the field. If we assume that all six of these closely related wasp species have a common mutation rate, we might estimate that value from the average u_{ihf} of wasp species associated with *F. citrifolia*, *F. nymphaefolia,* and *F. obtusifolia,* which is 2.84×10^{-5} for model 1P+S, and 2.70×10^{-5} for model 2P+S. Under both models, the q_{mut} estimates based on this average mutation rate fall well within the 95%CI of q_{field} for all wasp species except those associated with *F. popenoei A and B* (much too low) and F. *citrifolia* (slightly too high).

I here used 95% confidence intervals for q_{field} produced by a bootstrap method. The 95% CI for q_{field} produced by the generalized linear model with binomial errors and a logit link described in (Jandér and Herre 2010) are wider, but do not change the results – q_{field} for wasps associated with *F. popenoei* are still much higher than what the models predict.

2c. Game theoretical model quantifying the level of benefits needed to explain the empirical level of P- wasps if there are benefits.

If the level of pollen-free wasps in natural populations cannot be explained by mutations, another possibility is that there is some fitness benefit of being a P- wasp that balances the costs. Frequency dependent selection could then help maintain an equilibrium frequency of P- wasps in the population. The P- morph could be either genetically determined (pure strategies), or a mixed behavioural strategy, or a chosen strategy under certain environmental conditions (Table 3.2). Here I will use a game-theoretic model, in which the fitness benefit of being P- is the only mechanism counteracting the observed fitness costs, to examine how large the benefit would need to be under pure or mixed strategies to explain the observed frequency of P- wasps in natural populations. I here assume that the system has reached an evolutionarily stable frequency of P- wasps.

The different types of benefits

To model the benefits, we need to know what possible fitness advantage wasps that do not pollinate (P-) could have over pollinating (P+) wasps. To pollinate a receptive fig, a P+ wasp must successfully complete a number of steps: 1) collect pollen from her natal fig and securely place it in her pollen pockets, 2) carry the pollen in flight from her natal tree to the receptive tree, 3) carry the pollen while walking around inside the receptive fig, and 4) actively distribute pollen on the stigmas of flowers in the receptive fig using her front legs. At each of these steps, there are possible fitness advantages for P- wasps, indicated and numbered in figure 3.5 at the appropriate place. Different paths leading to different types of P- wasps are labeled by Greek letter in figure 5, and as subset Greek letters in the text, eg P-$_{\alpha,\beta}$ for a wasp that does not collect

pollen (α) and does not do pollination movements (β). Because I do not know what the true benefits are (if any), I here try to create an inclusive list of possible scenarios that could incur benefits.

Step 1:

Benefit A1. P-$_{\alpha}$ wasps do not spend time searching for and collecting pollen, and can therefore emerge from the fig ahead of the P+ wasps. Since predatory ants often wait by exit holes, and are more likely to be present the longer a fig has been open (Schatz and Hossaert-McKey 2003; Jandér personal observation), leaving the fig early would decrease the risk of being eaten, and therefore increase the relative chance of arriving at a receptive tree.

Benefit A2. P-$_{\alpha}$ wasps do not spend time searching for and collecting pollen, and therefore minimize the time that they are trapped inside the fig with males. Male wasps could accidentally damage the delicate females while searching for or fighting over access to females, especially when the more aggressive males of parasitic wasp species are present (Murray 1987; Pereira and Prado 2005; Pereira and Prado 2008), and/or when male densities are higher (and relatedness lower) due to high foundress numbers (Frank 1985; Herre 1985).

Both benefits A1 and A2 would result in a higher likelihood for P- wasps of successfully arriving at a receptive tree.

Step 2:

Benefit A3. P-$_{\alpha}$ and P-$_{\beta}$ wasps do not carry pollen during their flight, and due to this lighter load they can fly farther before they die, thereby increasing their chances of reaching a receptive tree. Under this scenario, P- wasps enter the receptive fig with equal energy as a P+.

OR **Benefit B1**. P-$_{\alpha}$ and P-$_{\beta}$ wasps do not carry pollen during their flight, and due to this lighter load they have larger energy reserves than P+ wasps when they reach a receptive fig. Wasps in these species are often energy limited (Jandér unpublished data), so with more energy, P- wasps can lay more eggs before they die.

Step 3:

Benefit B2. It is theoretically possible that carrying pollen while the wasp is walking around inside the fig and ovipositing is costly. However, the cost of carrying the pollen weight while walking is probably negligible. Further, any differences between P+ and P- would diminish over time as P+ wasps deposit pollen. Probably carrying pollen would only be a noticeable cost if the pollen were somehow poisoning the wasp – but this seems theoretically unlikely since it is in the natal tree's interest that the wasp pollinates as much as possible. For these reasons this type of benefit will not be modeled here.

Step 4:

Benefit B3. P-$_{\gamma}$ wasps do not do the pollination movements required to move pollen from their pollen pockets to receptive flowers. These pollination movements take time (2-5% of the time

inside a receptive fig, Jandér unpublished data) that otherwise could have been used for laying more eggs (in many cases, wasps are energy limited, Jandér unpublished data).

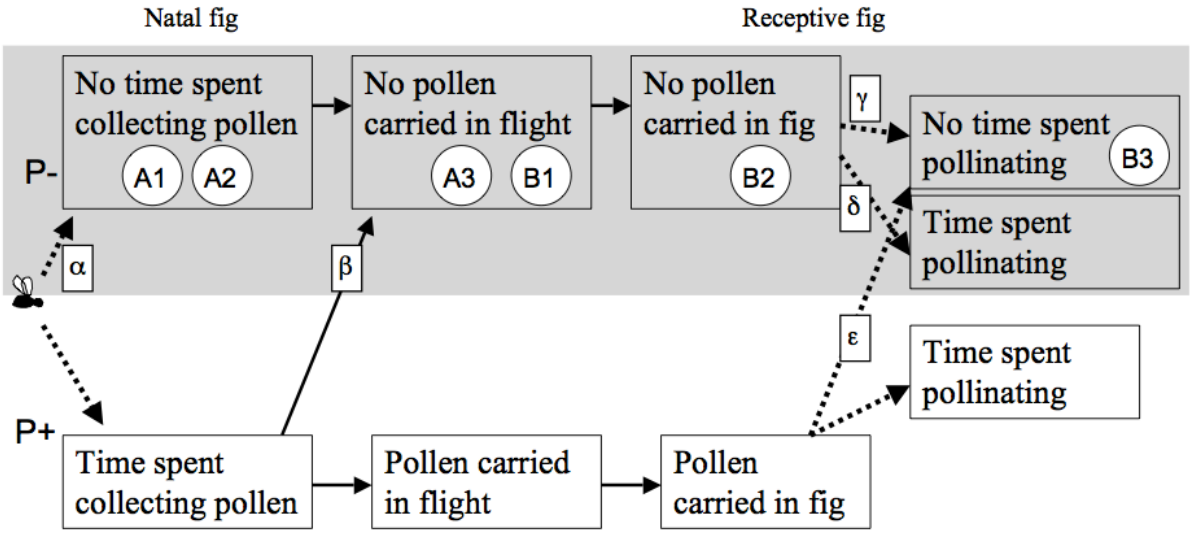

Figure 3.5. Flowchart depicting the four steps where pollen-free wasps may accrue benefits relative to pollen-carrying wasps. The different types of possible benefits at each step are labelled in white circles (see text). The grey shaded area represents steps resulting in a wasp that does not pollinate – each path that leads to this situation is labelled with a Greek letter (see text). Solid arrows represent non-choice transitions. Dotted arrows represent potential choices. The path labelled β indicates that the wasp was trying to collect pollen but failed for some reason.

Including benefits in the model

I will model two different types of benefits: A, those that lead to a higher likelihood of reaching a receptive fig (benefits A1, A2 and A3), and B, those that increase the number of eggs laid in the fig (benefits B1 and B3). For each of the models 1P+S and 2P+S, I will here evaluate each type of benefit, A and B, separately, to find out how large it would need to be to counteract the costs of being a P- wasp if that particular benefit were the only one operating. I will later discuss scenarios where multiple benefits act simultaneously.

Type A. *Benefits leading to a higher likelihood of reaching a receptive fig*

With type A benefits, P- wasps are more likely to arrive at a receptive tree than are P+ wasps. This could be because they are more likely to escape predatory ants upon emergence (benefit A1), because they are less likely to get damaged by male wasps inside the fig (benefit A2), or because, by carrying less weight, they can fly farther before they die (benefit A3). Building from the cost equations 1 and 2, the models used to estimate the levels of benefits are:

For the all-or-nothing 1P+S model:

$$W_{P-}(q) = k \cdot \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot \frac{t_n}{n} \cdot r_x \qquad (7a)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot \frac{t_n}{n} \cdot r_x \qquad (8a)$$

And for the gradual 2P+S model:

$$W_{P-}(q) = k \cdot \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot \frac{t_n}{n} \cdot r_{ax} \cdot r_{ox} \qquad (7b)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot \frac{t_n}{n} \cdot r_{ax} \cdot r_{ox} \qquad (8b)$$

where k is the relative likelihood of arrival at a receptive tree of a P- wasp compared to a P+ wasp, and q is the empirically determined proportion of P- wasps in natural populations (q_{field}).

Results

At the equilibrium proportion of P- in the population, the fitness of the two morphs will be equal (Maynard Smith 1982). Setting equations 7 & 8 equal, i.e. $W_{P-} = W_{P+}$, and inserting the appropriate species-specific values of q_{field}, f_n, t_n and r_x, I could calculate the needed species-specific equilibrium value of k. The level of benefit needed to explain the observed frequencies of P- wasps in natural populations varied dramatically across species. Model 1P+S could explain q_{field} in *F. popenoei* if P- wasps are only 4.5% more likely to arrive at receptive trees than are P+ wasps, whereas in *F. citrifolia,* P- wasps needed to be 211% more likely to arrive than P+ wasps (figure 3.6a). Under model 2P+S the equivalent values were 5.0% and 217% for *F. popenoei* and *F. citrifolia* respectively (Fig. 3.6b).

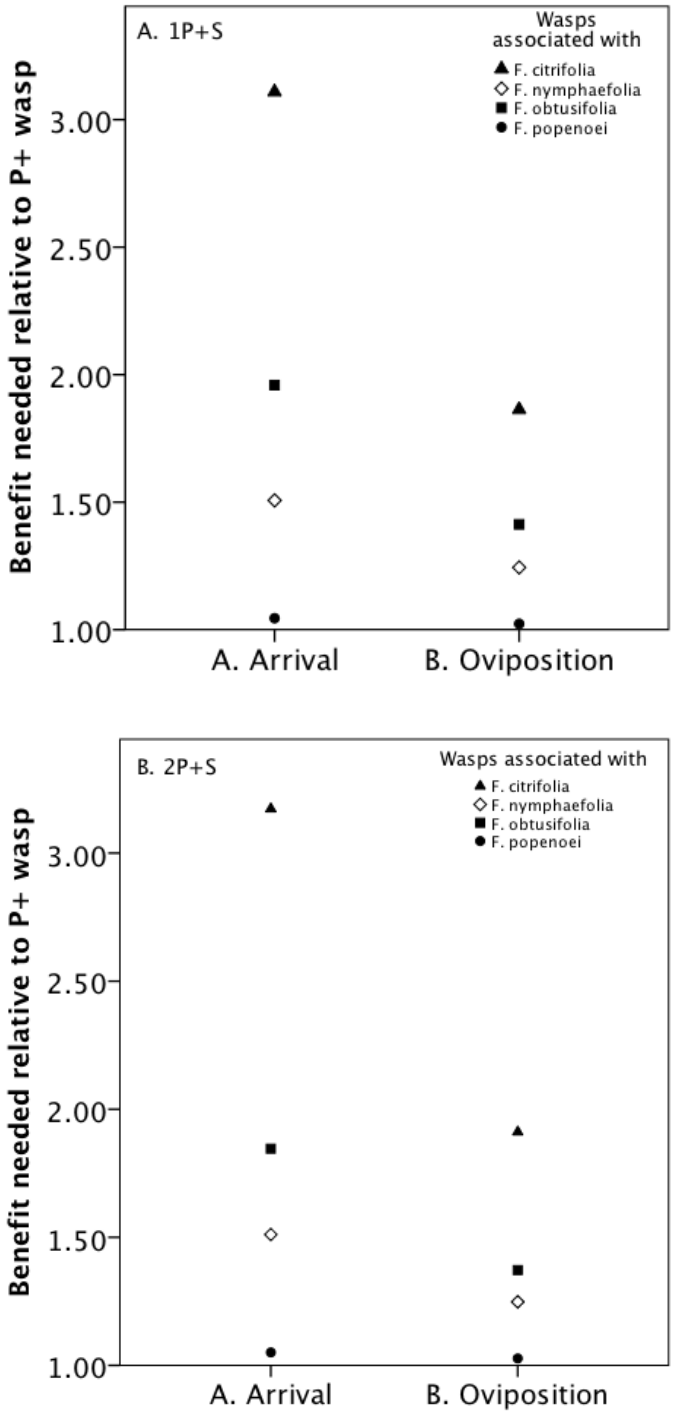

Figure 3.6. Estimated benefits (using cost – benefit models A (relative likelihood of arrival), and B (relative number of eggs laid) required for P- wasps relative to P+ wasps to explain the frequency of P- wasps found in natural populations. Calculations are based on model A) 1P+S, and B) 2P+S respectively.

Type B. *Benefits leading to relatively more eggs being laid in the fig*

With type B benefits, P- wasps lay more eggs than P+ wasps. This could be because they start with higher energy levels than P+ wasps (benefit B1), or because they can oviposit faster by omitting pollination movements (benefit B3). The oviposition rate in these species is not generally limited by egg numbers but by energy/lifespan (Jandér unpublished). Oviposition rates of these figwasps decrease approximately linearly over time (Raja *et al.* 2008; Jandér unpublished data), and can therefore be described by the linear equations

$$Ovipos.rate_{P+}(t) = b - at \qquad (9)$$

$$Ovipos.rate_{P-}(t) = c - dt \qquad (10)$$

where t is the time since entry into the fig (Fig. 3.7). I assume that $c \geq b$, and $c/d \geq b/a$. I further assume that the decay in pollination rate scales with the decay in oviposition rate (supported by Jandér, unpublished data) so that the relationship between the oviposition rates of P+ and P- wasps remains constant.

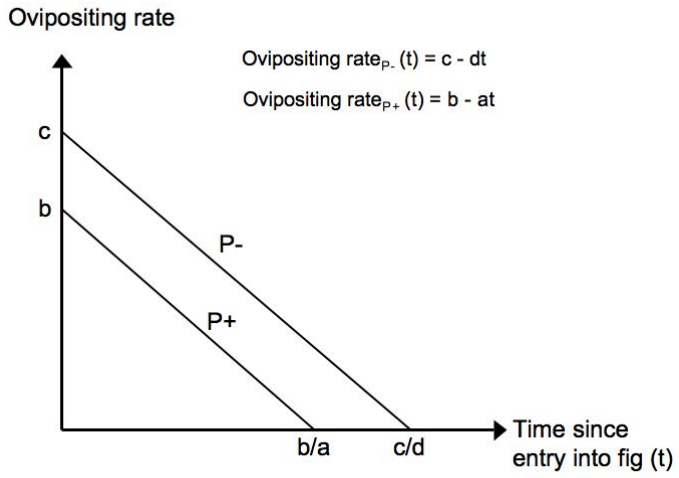

Figure 3.7. Theoretical example of how the oviposition rate of fig wasps decreases with time since entry into the fig fruit (see text).

The area under the curve represents the total number of eggs laid by a wasp, which here will be equated to the number of offspring the wasp would produce in that fig if there were no sanctions (wasps do not lay more than one egg per flower (Jousselin *et al.* 2001)). The relative number of offspring of a P- wasp compared to a P+ wasp, called *v*, can therefore be calculated as:

$$v = \frac{\frac{c^2}{2d}}{\frac{b^2}{2a}} \qquad (11)$$

We can simplify this equation by making the reasonable assumption that oviposition rates decrease similarly over time for P- and P+ wasps. Then, the initial oviposition rate for P+ and P- wasps differ, but the slope is the same (a = d in figure 3.7). Therefore, the simplified equation is

$$v = c^2/b^2 \qquad (12).$$

Density dependent competition

When oviposition sites (empty flowers) are abundant, energy/lifespan will be the factor limiting the total number of eggs laid before wasps die. However, with increasing number of foundresses (n) inside a fig, oviposition sites will become limiting. This can be seen in figs collected in nature, as total wasp offspring number from a fig first increases with increasing number of foundresses, then saturates at a species-specific maximum (Herre 1989). In this model, the total number of wasp offspring from a fig (t_n) for a given n will be allowed to increase up to the species-specific maximum t_{max} (estimated empirically). In a fig with low n, the faster P- wasps can therefore lay more eggs before they die, increasing t_n over that of a fig with only P+ foundresses. Once n is so high so that t_{max} is reached, P- and P+ wasps compete for a limited number of oviposition sites, and faster P- wasps then get a relatively larger proportion of t_{max}. Separate formulas will therefore be used for below-max n and at-max n. For example, in *F. popenoei*, n = 1-4 are below max, and $n \geq 5$ are at max (Herre 1988; Herre 1989).

For the all-or-nothing 1P+S model:

$$W_{P-}(q) = \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot r_x \cdot z_{nP-} \qquad (13a)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot r_x \cdot z_{nP+} \qquad (14a)$$

And for the gradual 2P+S model:

$$W_{P-}(q) = \sum_{n=1}^{n=m} \sum_{x=0}^{x=(n-1)} f_n \cdot \frac{(n-1)!}{(n-1-x)!x!} \cdot q^{(n-1-x)} \cdot (1-q)^x \cdot r_{ax} \cdot r_{ox} \cdot z_{nP-} \qquad (13b)$$

$$W_{P+}(q) = \sum_{n=1}^{n=m} \sum_{x=1}^{x=n} f_n \cdot \frac{(n-1)!}{(n-x)!(x-1)!} \cdot q^{(n-x)} \cdot (1-q)^{(x-1)} \cdot r_{ax} \cdot r_{ox} \cdot z_{nP+} \qquad (14b)$$

Where for both models:

$z_{nP-} = \frac{v \cdot t_n}{n}$ (equation 15) for all n before the species specific t_{max} is reached, and

$z_{nP-} = \frac{v \cdot t_{max}}{((n-x) \cdot v + x)}$ (equation 16) for all n once t_{max} is reached.

Conversely,

$z_{nP+} = \frac{t_n}{n}$ (equation 17) for all n before the species specific t_{max} is reached, and

$z_{nP+} = \frac{t_{max}}{((n-x) \cdot v + x)}$ (equation 18) for all n once t_{max} is reached.

Results

At the equilibrium proportion of P- in the population, the fitness of the two morphs will be equal (Maynard Smith 1982). Setting equations 13 & 14 equal, i.e. $W_{P-} = W_{P+}$, using equations 15-18 where appropriate, and inserting the species-specific values of q_{field}, f_n, t_n and r_x, I could calculate the species-specific equilibrium value of c/b. Again, the level of benefit needed to explain the frequency of P- wasps in natural populations varied across species. For type B benefits (oviposition rate) model 1P+S could explain q_{field} if P- wasps in *F. popenoei* had an initial oviposition rate only 2.3% faster than P+ wasps, whereas in *F. citrifolia* P- wasps needed to oviposit 86% faster (Fig. 3.6a). Under model 2P+S, the corresponding values were 2.7% and 91% (Fig. 3.6b).

3. Discussion

The results from the mutation selection balance model indicate that the mutation rates needed to explain the frequency of P- wasps found in the field are implausibly high for wasps associated with *F. popenoei*, but plausible for wasps associated with *F. citrifolia, F. nymphaefolia,* and *F. obtusifolia.* Consistent with this prediction, the results from the game-theoretical cost-benefit balance model indicate that the relative benefit levels needed to explain the frequency of P- wasps found in the field are implausibly high for wasps associated with *F. citrifolia, F. obtusifolia* and *F. nymphaefolia*, but plausible for those associated with *F. popenoei*. These results suggest that the mechanisms that maintain the behavioural polymorphism differ across wasp species.

Cost-benefit balance most likely in F. popenoei wasps

The models suggest that the observed frequencies of P- morphs in the two wasp species associated with *F. popenoei* are maintained by a balance between frequency-dependent costs due to tree-imposed sanctions and relative fitness benefits for P- wasps, which might accrue from increases in factors such as likelihood of reaching a receptive fig (type A) or number of eggs laid (type B). In theses species, P- wasps are predicted to be 4-5% more likely than P+ wasps to reach a flowering fig if only type A benefits are in effect. Reaching benefits of this size does not seem impossible by, for example, quickly abandoning crowded figs to avoid bodily damage by males (benefit A2), or carrying less weight in flight, which could increase flight duration (benefit A3). Field-based information regarding the presence and/or size of such benefits is currently lacking. If benefits instead are of type B, P- wasps are predicted to lay, on average, 2-3% more eggs than P+ wasps. Should wasps not perform pollination movements they would save 2-5% of time inside a fig (Jandér unpublished); since wasps of these species often are limited by time rather than by egg availability, that behaviour would increase the number of eggs that a foundress can lay (benefit B3). Another possibility is that wasps save energy by not carrying the weight of pollen in flight, and therefore enter a fig with more energy with which they can lay more eggs before they die (benefit B1).

Combinations of benefits are entirely possible, both within benefit types and across them. For example, a P- wasp might both benefit from avoiding ant predation (benefit A1) and by carrying less weight in flight (benefit A3), both of which may increase the likelihood of reaching a flowering fig (*k*). The estimated *k* is then made up of both types of benefits (at undetermined proportions), which requires each individual benefit to contribute less than would be needed if

benefits were acting singly. At one extreme, a "maximum cheater" P-$_{\alpha\gamma}$ wasp would neither collect pollen nor do pollination movements, and could therefore incur the full set of type A and type B benefits. However, wasps that skip only some steps in the pollination chain could also incur benefits. An "incomplete cheater" P-$_{\alpha\delta}$ wasp (not collecting pollen but doing pollination movements) could theoretically get all benefits except the time-savings of not depositing pollen (type B3). Likewise, a "failed pollinator" P-$_{\beta\delta}$ wasp (trying but failing to collect pollen then doing pollination movements) could nevertheless benefit from not carrying the pollen weight in flight (benefit A3 or B1).

In P- wasps associated with *F. citrifolia, F. nymphaefolia* and *F. obtusifolia*, the cost-benefit balance models predict that P- wasps would need to be 50% (*F. nymphaefolia*) to 210% (*F. citrifolia*) more likely than P+ wasps to arrive at receptive figs (type A benefits), or would need to lay on average 24% (*F. nymphaefolia*) to 86% (*F. citrifolia*) more eggs than P+ wasps (type B benefits). Because field-based information regarding the presence and/or size of benefits is currently lacking, I am not able to test the model predictions against field-based data for the different species. However, it seems unlikely that P- wasps would be able to incur such large benefits as are predicted for wasps associated with *F. citrifolia, F. nymphaefolia* and *F. obtusifolia*. In contrast, the benefit levels predicted for wasps associated with *F. popenoei*, 2-5%, seem quite plausible.

With the results from this paper alone it is not possible to determine whether the different wasp morphs associated with *F. popenoei* are more likely to be pure strategies (genetic polymorphism)

or mixed strategies (genetic monomorphism). The heritability of the P- trait is being studied; the results will help to distinguish between these two alternatives. If the P- trait is not strictly genetically determined, an alternative mechanism to mixed strategies (IIA, table 3.2) is that being a P- wasp might be a chosen strategy under certain environmental conditions (IIB, table 3.2) (Gross 1996). An ongoing study is comparing the environmental conditions (density of males, density of parasitic males, pollen availability etc.) within figs that produce a high proportion of P- with those within figs that produce only P+ wasps, which will give clues as to whether environmental conditions affect the proportion of P- wasps emerging from a fig. Thus, ongoing and future studies will shed further light on which mechanism maintains the P- trait in wasps associated with *F. popenoei.*

Mutation-selection balance most likely in other species

The models suggest that the observed frequencies of P- wasps in all wasp species associated with *F. citrifolia, F. nymphaefolia*, and *F. obtusifolia* are maintained by a balance between mutations and costs due to tree-imposed sanctions. The predicted mutation rates vary between 6×10^{-6} (wasps associated with *F. citrifolia*) to 4×10^{-5} (wasps associated with *F. obtusifolia*). The average mutation rate of 2.8×10^{-5} produces estimates of the prevalence of P- wasps that are consistent with field-recorded values for all the associated wasp species. Is this a reasonable mutation rate? Mutation rates per locus per generation are commonly estimated to be 10^{-6} to 10^{-5} for eukaryotes (Falconer and Mackay 1996; Nachmann and Crowell 2000; Haag-Liautard *et al.* 2007; Lynch 2010). The estimated mutation rates of 6×10^{-6} to 4×10^{-5} therefore do not seem unreasonable, especially considering that these would be high-end estimates. It is likely that not just one, but multiple genes affect the pollination behaviour of fig wasps. Loss of function of any

one of these genes could create P- wasps. Therefore, the mutation rate needed to explain the field-observed prevalence of P- wasps is in reality likely to be lower than what these single-gene models predict. Nevertheless, the predicted mutation rates needed by wasps associated with *F. popenoei* (4×10^{-4} to 8×10^{-4}) are an order of magnitude higher and seem implausibly high.

An alternative to the mutation-selection mechanism is that P- wasps are environmentally caused mistakes that are not heritable and experience no benefits, only costs. For example, under some circumstances individual fig fruits might not contain enough pollen for all female wasps emerging from that fig. However, preliminary data from *F. popenoei* suggest that there is no lack of pollen in figs producing a high proportion of P- wasps (Jandér unpublished). Because P- wasps are so rare in wasps associated with the other fig species, I have been unable to conduct similar investigations for other fig species.

Conclusion

In actively pollinated fig species where sanctions are weak, and unlikely due to high average number of foundresses, the costs of being a P- wasp are small. In such cases, the relative benefits needed to balance those costs are relatively low. It further seems plausible that there are some benefits associated with being a P- wasp, although these benefits are probably rather small. If these small benefits nevertheless increase the fitness of P- wasps over that of P+ wasps, P- wasps will spread in the population, past the frequency that could be explained by the base-level mutation rate alone, until an equilibrium frequency is reached. This scenario seems to be the case for wasps associated with *F. popenoei*. On the other hand, if strong and likely sanctions impose fitness costs that outweigh the small fitness benefits of being a P- wasp, then P- wasps should

theoretically be absent if their frequency is governed only by their relative fitness. Presumably, however, mutations continually add new P- wasps to wasp populations. Therefore, in wasp species where the benefits of being a P- wasp cannot outweigh the costs, the prevalence of P- wasps should be dictated by a balance between net costs and mutation rates. This seems to be the case in wasps associated with *F. citrifolia, F. nymphaefolia,* and *F. obtusifolia.* In the six wasp species studied here, the conclusions did not depend on whether sanctions act only on completely unpollinated figs (1P+S), or if wasps in poorly pollinated figs also suffer mild sanctions (2P+S).

This study exemplifies how mathematical models combined with empirical data can help distinguish among alternate hypotheses. Although the data in this paper were obtained from a few species of figs and their pollinating wasps, the general conclusions are applicable both to other actively pollinated fig species, and to other mutualisms with sanctions and costly services. Frequencies of uncooperative symbionts may be governed by mutation rates in species where hosts can impose sufficiently strong or directed sanctions. On the other hand, in hosts where sanctions are weak or imprecise, uncooperative symbionts may proliferate until they reach the equilibrium frequency at which costs and benefits of not being cooperative are balanced.

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